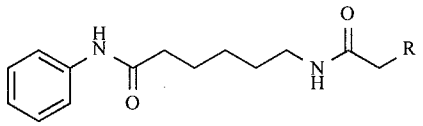


30: R = -SH, IC_{50} (HDACs) = 0.39 μM
31: R = -OH, IC_{50} (HDACs) > 100 μM
32: R = $-NH_2$, IC_{50} (HDACs) > 100 μM

Fig. (16). Hetero atom-containing substrate analogues designed on the basis of the deacetylation mechanism.

HDAC inhibitors (Fig. (**17**)b). As we had expected, strong inhibition was observed with mercaptoacetamide **30**, while lysine substrate, with an inhibition constant (*Ki*) of 0.78 μM on Lineweaver-Burk plots. The low energy conformation of **30** docked in the model based on the crystal structure of HDAC8 was calculated. An inspection of the HDAC8/**30** complex showed that the sulfur atom and oxygen atom of **30** were located 2.44 Å and 2.04 Å from the zinc ion, respectively, and that a water molecule, which is required for the deacetylation of acetylated lysine substrate, was positioned 4.95 Å apart from the zinc ion (Fig. (**18**)). This calculation suggests that **30** inhibits HDACs by chelating the zinc ion in a bidentate fashion through its sulfur and oxygen atoms, and by removing a water molecule from the zinc and the reactive site of the deacetylation, without being hydrolyzed by HDACs.

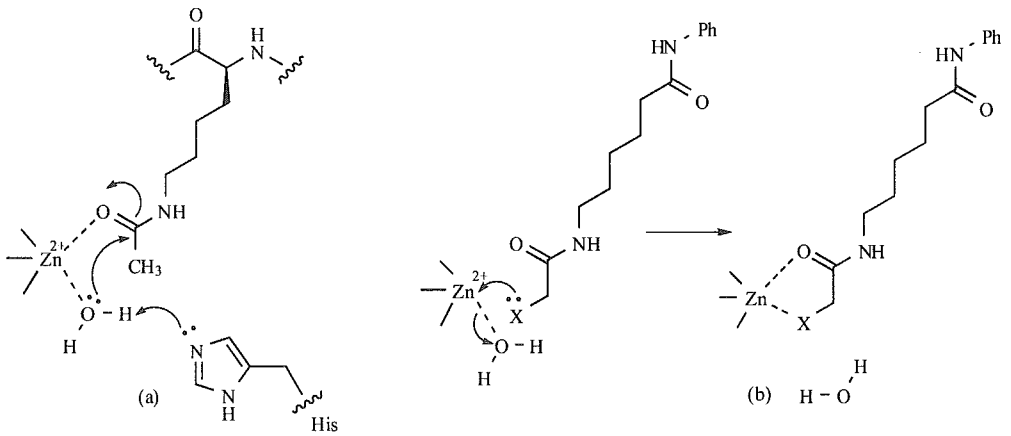


Fig. (17). Mechanism proposed for the deacetylation of acetylated lysine substrate (a), and model for the binding of hetero atom-containing substrate analogues to zinc ion (b).

31 and **32** did not possess inhibitory activity. Mercaptoacetamide **30** exhibited an IC_{50} of 0.39 μM and was found to be a competitive inhibitor versus acetylated

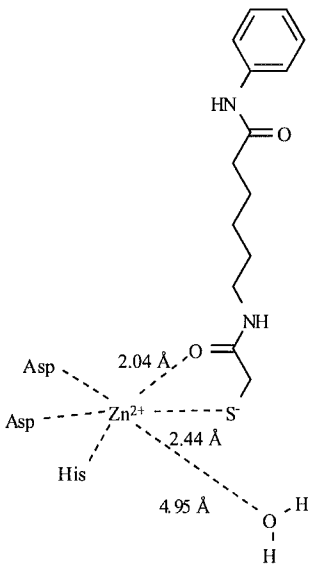
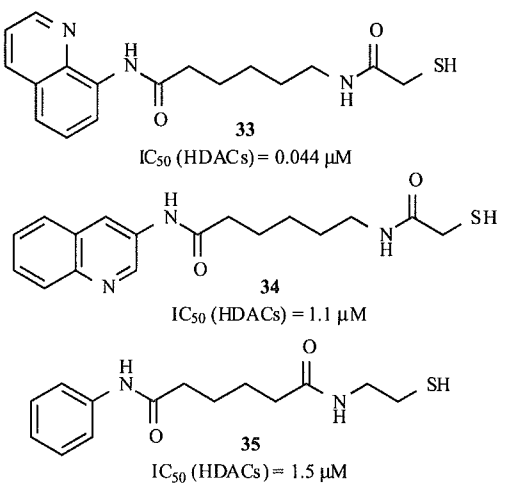


Fig. (18). Low energy conformation of **30** (IC_{50} = 0.39 μM) docked in the HDAC8 catalytic core.

Potent HDAC inhibitors with a mercaptoacetamide moiety have recently been reported independently by two research groups. Chen and co-workers have identified 8-quinolynyl compound **33** (IC_{50} = 0.044 μM) [72], and Anandan and co-workers have reported 3-quinolynyl compound **34** (IC_{50} = 1.1 μM) [73] (Fig. (**19**)). In addition, mercaptoethylamides such as **35** have also been reported to



33
IC_{50} (HDACs) = 0.044 μM

34
IC_{50} (HDACs) = 1.1 μM

35
IC_{50} (HDACs) = 1.5 μM

Fig. (19). Mercaptoamide-based HDAC inhibitors.

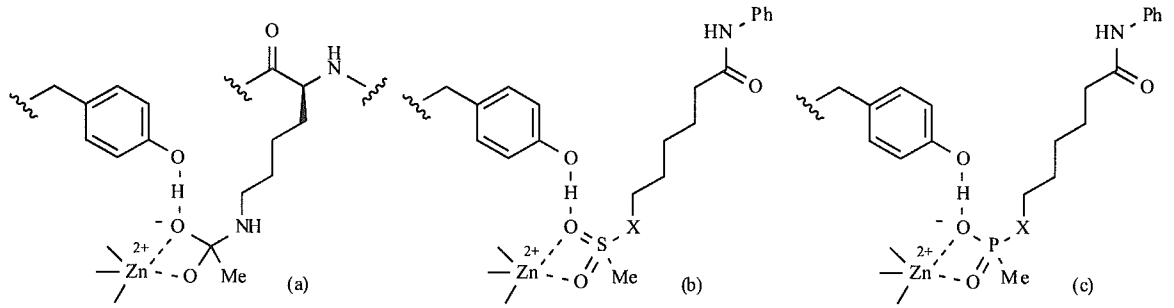
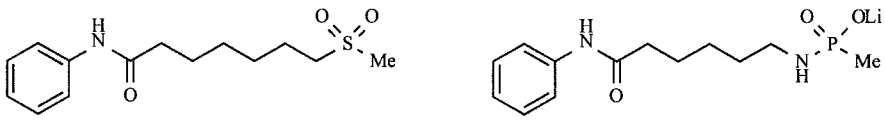


Fig. (20). Transition state proposed for HDACs (a), and models for the binding of sulfone derivatives (b) and phosphone derivatives (c).

inhibit HDACs with IC_{50}s of 1–100 μM [73]. Since mercaptoacetamides are reported as potent, long-lasting, and less toxic matrix metalloproteinase inhibitors [74, 75], they could be more improved HDAC inhibitors.

Sulfones and Phosphones as Transition State Analogues

Transition state (TS) analogue inhibitors have been reported independently by us [71] and Etzkorn et al [76]. The TS of HDAC deacetylation was estimated to include a tetrahedral carbon [18-20] (Fig. (**20**)a) as with other zinc proteases [77]. Sulfone and phosphone derivatives could be TS analogue inhibitors because they have strong similarity with the TS of amide bond hydrolysis (Fig. (**20**)b,c). Among these TS analogues, sulfone **36** [71] and phosphonamidate **37** [76] (Fig. (**21**)) inhibited HDACs at high micromolar concentrations. In addition, the antiproliferative activity of **37** against A2780 cancer cells (IC_{50} = 120 μM) was comparable to that of SAHA (IC_{50} = 150 μM).



36
IC_{50} (HDACs) = 230 μM

37
IC_{50} (HDAC8) = 280 μM
IC_{50} (HDACs) = 570 μM
IC_{50} (HDLP) = 330 μM

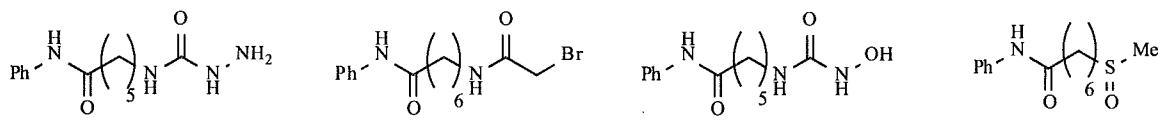
Fig. (21). Transition state analogue HDAC inhibitors.

Miscellaneous

We recently presented SAHA-based non-hydroxamates **38**–**41** (Fig. (**22**)), as HDAC inhibitors, which were identified by structure-based drug design [68, 78, 79]. These compounds displayed anti-HDAC activities with IC_{50}s of 14–150 μM. Although their inhibitory activities are weaker than that of SAHA, sulfoxide **41** showed a certain type of selectivity (see the section Isozyme Selectivity).

Design and synthesis of 3-(4-aroyl-1*H*-2-pyrrolyl)-2-propene derivatives were reported by Mai et al [44]. Among them, barbiturate **42**, thiobarbiturate **43**, nitrile **44** and amidine **45** exhibited inhibitory activities against maize HDAC2 with IC_{50}s of 23–100 μM (Fig. (**23**)).

Some natural products have been reported to inhibit HDACs (Fig. (**24**)). Schreiber and co-workers reported that depudecin **46**, a metabolite isolated from the fungus Alternaria brassicicola, inhibits HDAC1 with an IC_{50} of 4.7 μM [80]. The mechanism of action of depudecin **46** is yet unclear, but it may inhibit HDACs irreversibly like trapoxin B **8**. Crews and co-workers isolated novel disulfide bromotyrosine derivatives such as psammaplins F **47** from the sponge *Pseudoceratina purpurea*, which were found to be highly potent HDAC inhibitors (IC_{50} of **47** = 2.1 nM) [81]. Interestingly, some of the psammaplins also showed inhibitory activity against semipurified DNA methyltransferase I. It is not known exactly how psammaplins inhibit these enzymes. Fusetani and co-workers isolated new cyclostellettamines such as dehydrocyclostellettamine D **48** from a marine sponge of the



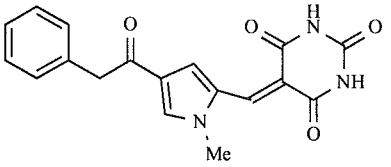
38
IC_{50}(HDACs) = 150 μM

39
IC_{50} (HDACs) = 14 μM

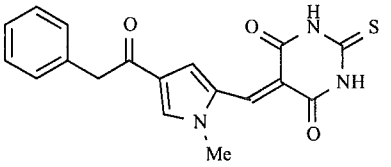
40
IC_{50} (HDACs) = 80 μM

41
IC_{50} (HDACs) = 48 μM

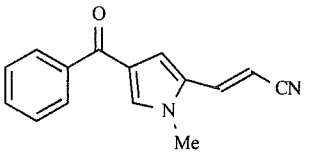
Fig. (22). SAHA-based non-hydroxamate HDAC inhibitors identified by structure-based drug design.



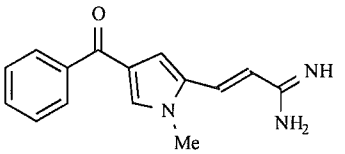
42
IC_{50} (maize HDAC2) = 100 μM



43
IC_{50} (maize HDAC2) = 85 μM



44
IC_{50} (maize HDAC2) = 27 μM



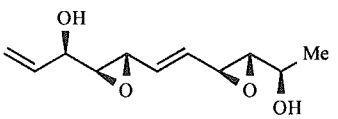
45
IC_{50} (maize HDAC2) = 23 μM

Fig. (23). 3-(4-Aroyl-1*H*-2-pyrrolyl)-2-propene derivatives.

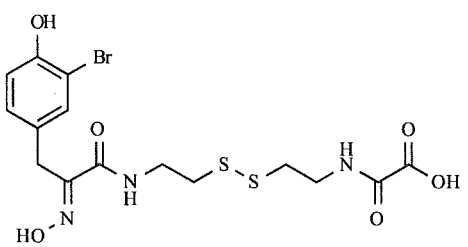
genus *Xestospongia* [82]. These cyclostellettamines were found to inhibit HDACs derived from K562 human leukemia cells with IC_{50}s ranging from 17 to 80 μM. However, they showed cytotoxicity against P388, HeLa, and 3Y1 cells at lower concentrations (IC_{50} of 0.6–11 μM). These results indicate that these cyclostellettamines have biological activities other than inhibition of HDACs.

ISOZYME SELECTIVITY

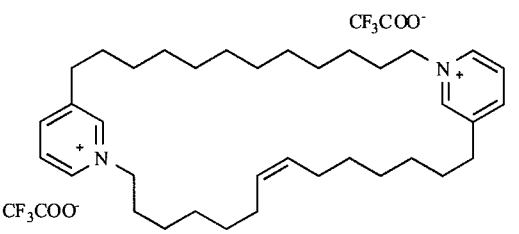
The HDAC family is divided into two categories, zinc-dependent enzymes and NAD^+-dependent enzymes [1, 83]. The HDAC inhibitors described above are inhibitors of zinc-dependent enzymes. Thus far, eleven zinc-dependent HDAC family members have been identified and they are classified into two classes (Class I and Class II) based on sequence similarity (Table 1). Class I HDACs include HDAC1, HDAC2, HDAC3 and HDAC8, and show homology to the yeast protein RPD3. Class II HDACs include HDAC4, HDAC5, HDAC6, HDAC7, HDAC9 and HDAC10, and are homologous to the yeast enzyme HDA1. HDAC11, the most recently identified member, most likely belongs to Class I, although the sequence similarity is low [84]. Class I HDACs are expressed in the nuclei of most cell lines and are involved in the transcriptional repression of a number of genes [85-87]. On the contrary, the localization of Class II enzymes is not limited to the nucleus. They are also expressed in cytoplasm [88] and are associated with muscle differentiation block [89] and microtubule stability (see below). HDACs have been reported to be linked with carcinogenesis [90-92], while tissue-specifically expressed HDAC isozymes are known to play important roles in the generation and differentiation of normal tissues [89, 93-95].



46: depudecin
IC_{50} (HDAC1) = 4.7 μM



47: psammaplin F
IC_{50} (HDACs) = 2.1 nM



48: dehydrocyclostellettamine D
IC_{50} (HDACs) = 17 μM

Fig. (24). Structures of depudecin **46**, psammaplin F **47** and dehydrocyclostellettamine D **48**.

Table 1. Classification of Zinc-Dependent HDACs [1, 83-89]

HDAC	localization	function
Class I (RPD3 homologue)		
HDAC1	nucleus	transcription repression
HDAC2	nucleus	transcription repression
HDAC3	nucleus, cytoplasm	transcription repression
HDAC8	nucleus	transcription repression
HDAC11	nucleus	unknown
Class II (HDA1 homologue)		
HDAC4	nucleus, cytoplasm	transcription repression
		muscle differentiation block
HDAC5	nucleus, cytoplasm	transcription repression
		muscle differentiation block
HDAC6	cytoplasm	regulation of microtubule stability
		and function
HDAC7	nucleus, cytoplasm	transcription repression
		muscle differentiation block
HDAC9	nucleus, cytoplasm	unknown
HDAC10	cytoplasm	unknown

Therefore, isozyme-selective HDAC inhibitors are considered to be useful not only as tools for probing the biology of HDAC isozymes but as drugs with low toxicity. Interestingly, many non-hydroxamate HDAC inhibitors have shown isozyme selectivity. In this section, we describe the selectivity of non-hydroxamate HDAC inhibitors in enzyme assays and in cellular assays.

Table 2. Inhibitory activities of HDAC inhibitors against HDAC1 and HDAC6 [98]

Inhibitors	mean IC_{50} (nM)		HDAC6 IC_{50}/HDAC1 IC_{50}
	HDAC1	HDAC6	
TSA (**3**)	19	14	0.74
trapoxin B (**8**)	0.13	74	570
FK228 (**28**)	36	14,000	390
MS-275 (**10**)	250	>10,000,000	>40,000
sodium bytyrate (**4**)	170,000	>10,000,000	>59

Of the eleven HDAC isozymes, HDAC6 has been studied the most. HDAC6 is unique in that it is essentially cytoplasmic and has the interesting property of being capable of nucleocytoplasmic shuttling. In addition, HDAC6 is predominantly expressed in the testes, and is speculated to be involved in the differentiation of normal tissues [96]. It has been also reported that HDAC6 expression is correlated with breast cancer [97]. Thus, HDAC6-insensitive inhibitors could be medicines with less toxicity. Yoshida and co-workers investigated the in vitro inhibitory effects of a wide variety of HDAC inhibitors including non-hydroxamates on HDAC1 and HDAC6 [98]. As shown in Table 2, TSA **3**, a representative hydroxamate inhibitor, inhibited both HDAC1 and HDAC6. Although trapoxin B **8**, an inhibitor bearing epoxyketone, strongly inhibited HDAC1 at subnanomolar concentrations, it failed to inhibit HDAC6 at these concentrations. Like trapoxin B **8**, other non-hydroxamates such as sodium butyrate **4**, MS-275 **10** and FK228 **28** inhibited HDAC1 more than HDAC6.

HDAC6 has also been reported to be an α-tubulin deacetylase (TDAC) [99]. Unlike other HDAC isozymes, HDAC6 has two catalytic domains, one for histones (the HDAC site) and the other for α-tubulin (the TDAC site) [100, 101]. Inhibition of the TDAC site of HDAC6, which is responsible for the deacetylation of α-tubulin, is usually assessed by the accumulation of acetylated α-tubulin in a cellular assay (Western blot analysis) after treatment with inhibitor. Most hydroxamate HDAC inhibitors such as SAHA **1** and TSA **3** have been reported to cause both histone hyperacetylation and α-tubulin acetylation in cells, which suggests that these hydroxamates do not discriminate between TDAC (HDAC6) and the other isozymes [98, 99, 102, 103]. In contrast, the non-hydroxamate HDAC inhibitors that have been assessed for their effect on α-tubulin acetylation status do not cause an accumulation of acetylated α-tubulin and are presumably not inhibitors of the TDAC site of HDAC6 (Table 3). Schreiber and co-workers were the earliest to disclose non-hydroxamate HDAC inhibitors which are insensitive to the TDAC site of

Table 3. Effect on Acetylation of α-Tubulin

Inhibitors	Ac-α-tubulin[a]	References
Hydroxamic acids		
SAHA (**1**)	(+)	[103]
TSA (**3**)	(+)	[98, 99, 101, 102]
Non-hydroxamates		
histacin (**14**)	(-)	[28]
PAOA (**15**)	(-)	[56]
MS-275 (**10**)	(-)	[63]
sodium bytyrate (**4**)	(-)	[99]
trapoxin B (**8**)	(-)	[98, 99]
FK228 (**28**)	(-)	[102, 104]
α-ketoamide (**19**)	(-)	[63]
sulfoxide (**41**)	(-)	[79]

[a] (+) indicates an accumulation of acetylated α-tubulin while (-) indicates no effect on acetylation.

HDAC6. A multidimensional, high-throughput, cell-based screening of 7,392 small molecules prepared using a one-bead/one-stock solution strategy led to the identification of histacin **14**, which is inactive toward TDAC (HDAC6) in cells [28, 55]. These workers have also extended this finding by creating SAHA/*o*-aminoanilide hybrids such as PAOA **15** that inhibit nuclear HDAC isozymes over cytoplasmic TDAC (HDAC6) [56]. Another *o*-aminoanilde inhibitor MS-275 **10** was shown not to increase the acetylation state of α-tubulin, either [63]. It has been reported that the carboxylic acid sodium butyrate **4**, the epoxyketone trapoxin B **8** and the disulfide FK228 **28** show greater inhibition of nuclear HDACs than TDAC (HDAC6) in cells [98, 99, 102, 104]. Glaser and co-workers at Abbott found that α-ketoamide HDAC inhibitor **19** was selective for the TDAC site of HDAC6 in cells, although **19** did not distinguish between HDAC1/2 and HDAC6 in enzyme assays [63]. Very recently, we have reported that sulfoxide **41** showed great HDAC/TDAC selectivity in cellular assays [79].

It is surprising that non-hydroxamate HDAC inhibitors discriminated between HDAC6 and other isozymes in enzyme assays and cellular assays because the amino acid residues surrounding the zinc ion in the active site of HDACs are highly conserved [13, 55]. It is still unclear why non-hydroxamates distinguish well between HDAC6 and other isozymes. However, the nature of ZBG of HDAC inhibitors is an important factor determining the selectivity for HDAC6. As mentioned above, HDAC6 is estimated to be responsible for the differentiation of normal tissues such as the testis. Furthermore, the reversible acetylation of α-tubulin, which is partly regulated by HDAC6, is involved with microtubule stability [99]. Therefore, the isozyme selectivity of non-hydroxamates has important clinical implications for the development of new medicines without side effects caused by interference with nuclear homeostasis and microtubule dynamics associated with HDAC6.

PERSPECTIVE

Worldwide research on HDAC has led to the discovery of a number of structurally diverse inhibitors of HDACs. The first generation of HDAC inhibitors including SAHA, NVP-LAQ824 and MS-275 are currently in clinical trials. There is now strong evidence from clinical studies that these HDAC inhibitors are effective. The therapeutic potential is expected to increase when further positive clinical findings are unveiled. However, hydroxamic acid-based inhibitors have perceived liabilities such as poor pharmacokinetics, and the lack of isozyme selectivity of hydroxamates may cause severe toxicity. The discovery of non-hydroxamate HDAC inhibitors introduced in this review has provided the basis for the development of more improved HDAC inhibitors. Further study on non-hydroxamate HDAC inhibitors will also offer a basis on which to better design isozyme-selective inhibitors and to overcome the drawbacks of hydroxamates.

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Nitrogen-substitution effect on in vivo mutagenicity of chrysene

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Abstract

We have previously reported the in vivo mutagenicity of aza-polycyclic aromatic hydrocarbons (azaPAHs), such as quinoline, benzo[*f*]quinoline, benzo[*h*]quinoline, 1,7-phenanthroline and 10-azabenzo[*a*]pyrene. The 1,10-diazachrysene (1,10-DAC) and 4,10-DAC, nitrogen-substituted analogs of chrysene, were shown to exhibit mutagenicity in *Salmonella typhimurium* TA100 in the presence of rat liver S9 and human liver microsomes in our previous report, although DACs could not be converted to a bay-region diol epoxide, the ultimate active form of chrysene, because of their nitrogen atoms. In the present study, we tested in vivo mutagenicity of DACs compared with chrysene using the *lacZ* transgenic mouse (Muta™Mouse) to evaluate the effect of the nitrogen substitution. DACs- and chrysene-induced mutation in all of the six organs examined (liver, spleen, lung, kidney, bone marrow and colon). The mutant frequencies obtained with chrysene showed only small differences between the organs examined and ranged from 1.5 to 3 times the spontaneous frequency. The 4,10-DAC was more mutagenic than chrysene in all the organs tested. The highest *lacZ* mutation frequency was observed in the lung of 4,10-DAC-treated mice and it was 19 and 6 times the spontaneous frequency and the frequency induced by chrysene, respectively. The 1,10-DAC induced *lacZ* mutation in the lung with a frequency 4.3- and 1.5-fold higher than in the control and chrysene-treated mice, respectively, although the mutant frequencies in the other organs of 1,10-DAC-treated mice were almost equivalent to those of chrysene-treated mice. Not only chrysene but also DACs depressed the G:C to A:T transition and increased the G:C to T:A transversion in the liver and lung. These results suggest that the two types of nitrogen substitutions in the chrysene structure may enhance mutagenicity in the mouse lung, although they showed no difference in the target-organ specificity and the mutation spectrum.

Keywords: Aza-substitution; In vivo mutagenesis assay; Mutation spectrum

1. Introduction

We have been investigating the mutagenicity of aza-polycyclic aromatic hydrocarbons (azaPAHs) with

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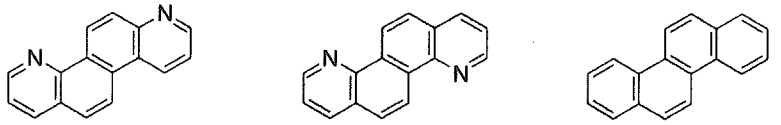

Fig. 1. Chemical structures of 1,10-DAC, 4,10-DAC and chrysene.

special attention to their metabolic activation mechanism. The 10-azabenzo[*a*]pyrene (10-azaBaP), a 10-aza-analog of benzo[*a*]pyrene (BaP), was reported to be as mutagenic as BaP in *Salmonella typhymurium* TA100 in the presence of PCB-treated rat liver S9 [1,2], although 10-azaBaP could not be converted to a bay-region diol epoxide, the ultimate mutagenic form of BaP [3,4], because of its nitrogen atom. We have previously reported that 10-azaBaP showed a higher mutagenicity than BaP in the Ames test using pooled human liver S9 [5]. However, in the in vivo mutagenesis assay system using the *lacZ* transgenic mouse (Muta^{TM}Mouse), 10-azaBaP was mutagenic only in the liver and colon and showed much less mutagenicity than BaP, which showed high mutagenicity in all of the organs tested [5]. Thus, 10-azaBaP interestingly showed differences in mutagenicity between the in vitro and in vivo assay systems.

We have also reported that quinoline, an aza-analog of naphthalene, one of simplest azaPAHs and a hepatocarcinogen [6,7], showed mutagenicity only in the liver of Muta^{TM}Mouse [8]. We also observed that it caused remarkable induction of G:C to C:G transversion [9] and suggested that it might be metabolically activated in the pyridine moiety to the ultimate mutagenic form [10]. Its active form was supposed to be an enamine epoxide (1,4-hydrated 2,3-epoxide), which would be responsible for the mutagenic modification of DNA [11–14]. Furthermore, three trycyclic azaPAHs, i.e., benzo[*f*]quinoline, benzo[*h*]quinoline and 1,7-phenanthroline, were shown to exhibit mutagenicity in Muta^{TM}Mouse in our previous report [15]. Benzo[*h*]quinoline and 1,7-phenanthroline were suggested to be converted to the ultimate genotoxic form in the pyridine moiety [16].

1,10-Diazachrysene (1,10-DAC) and 4,10-DAC are diaza-analogs of chrysene (Fig. 1), consisting of two quinoline moieties, and have structures similar to 10-azaBaP. We have previously reported that these DACs showed mutagenicity in Ames tests in the presence of rat liver S9 or human liver microsomes [17], although formation of the bay-region diol epoxide from DACs seemed impossible because of their nitrogen atoms. DACs have not been found in our living environments, but these are expected to be useful compounds to investigate the nature of mutagenicity in azaPAHs.

In the present study, we undertook to investigate the in vivo mutagenicity of DACs in comparison with chrysene by the in vivo mutation assay system using the *lacZ* transgenic mouse (Muta^{TM}Mouse) to evaluate the nitrogen-substitution effect in the chrysene skeleton on their mutagenicity.

2. Materials and methods

2.1. Materials

Chrysene (CAS Registry No. 218-01-9) and phenyl-β-D-galactoside (P-gal) were purchased from Sigma Chemical Co. (St. Louis, MO), proteinase K and olive oil from Wako Pure Chemicals (Osaka) and RNase from Boehringer Mannheim. The 1,10-DAC (CAS Registry No. 218-21-3) and 4,10-DAC (CAS Registry No. 218-34-8) were synthesized in this laboratory according to the reported methods [18].

2.2. In vivo mutagenesis assays using Muta^{TM}Mouse

2.2.1. Animals and treatments

Male Muta^{TM}Mice, at 7–8 weeks of age, were supplied by COVANCE Research Products (PA, USA) and acclimatized for 1 week before use. Chrysene and 4,10-DAC dissolved in olive oil (10 mL/kg body weight)

were injected intraperitoneally into four mice each at a single dose of 200 mg/kg once a week for 4 consecutive weeks (800 mg/kg in total). The 1,10-DAC dissolved in olive oil (10 mL/kg body weight) was injected into four mice at a single dose of 100 mg/kg similarly (400 mg/kg in total). Four control mice were given 10 mL olive oil/kg.

2.2.2. Tissues and DNA isolation

All mice were killed by cervical dislocation 7 days after the last administration of test chemicals. The liver, spleen, lung, kidney, bone marrow and colon were immediately extirpated, frozen in liquid nitrogen and stored at −80 °C until DNA extraction. The genomic DNA was extracted from each tissue by the phenol/chloroform method according to the Muta™Mouse/PS Mutation Assay Manual (Corning Hazleton, 1995). The isolated DNA, which was precipitated with ethanol, was air-dried and dissolved in an appropriate volume (20–200 μL) of TE-4 buffer (10 mM Tris–HCl at pH 8.0 containing 4 mM EDTA) at room temperature overnight. The DNA solution thus prepared was stored at 4 °C.

2.2.3. In vitro packaging

The lambda gt10/*lacZ* vector could be efficiently recovered by in vitro packaging reactions [19]. Our homemade packaging extract (HM) consisting of sonic extract (SE) of *Escherichia coli* NM759 and freeze–thaw lysate (FTL) of *E. coli* BHB2688 was prepared according to the method of Gunther et al. [20]. As a general procedure for handling the HM extract, approximately, 5 μg DNA was mixed with 15 μL of FTL and 30 μL of SE and incubated at 37 °C for 90 min. Then SE and FTL were added again and the mixture was incubated for another 90 min. The reaction was terminated by the addition of an appropriate volume of SM buffer (50 mM Tris–HCl at pH 7.5, 10 mM $MgSO_4$, 100 mM NaCl and 0.01% gelatin) and the mixture was stored at 4 °C. By this procedure, the lambda gt10 vector to form an infectious phage was efficiently rescued from genomic DNA.

2.2.4. Mutation assays

2.2.4.1. lacZ mutant frequency determination. The positive selection for *lacZ* mutants was performed as previously reported [21–23]. Briefly, the phage

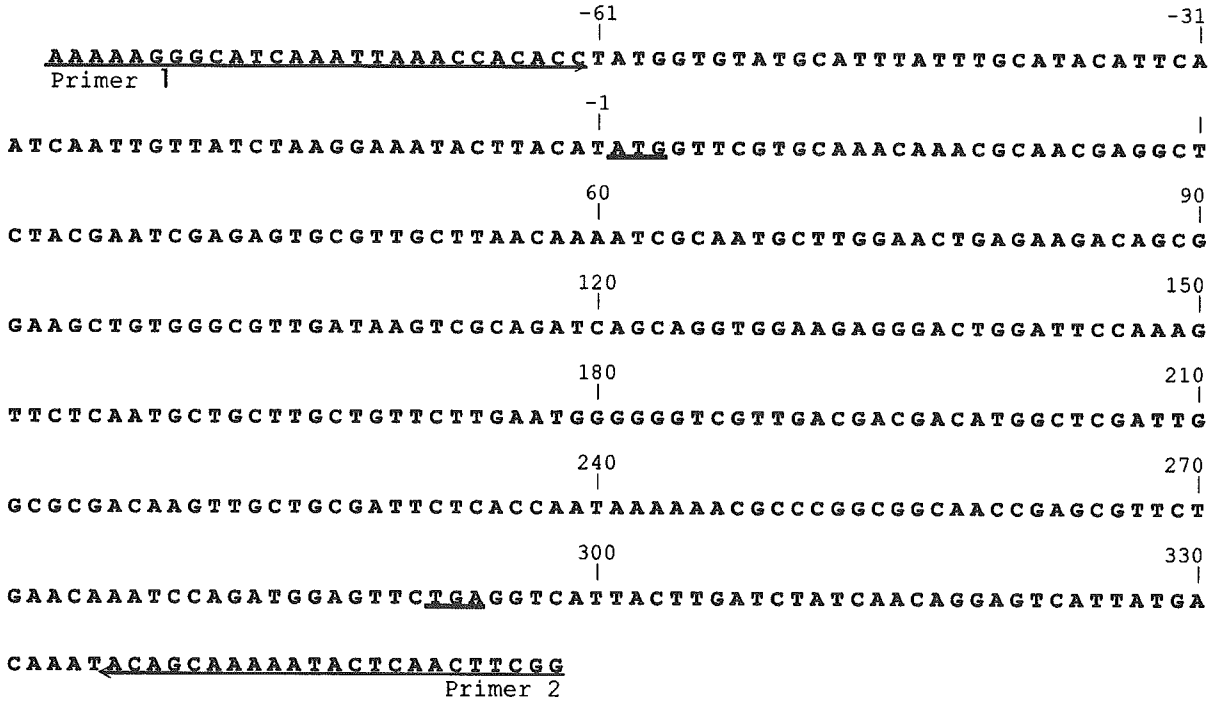

Fig. 2. Sequence map of the *cII* gene; primers used for PCR amplification and sequencing are shown by arrows. The PCR gives 446 bp products that involve the entire (294 bp) *cII* gene. Initiation and stop codons are underlined.

Table 1
Mutant frequencies induced by 1,10-DAC, 4,10-DAC and chrysene in six organs of Muta^{TM}Mouse

Tissue	Treatment	*lacZ* assay				*cII* assay			
		Individual animal data			Average ± S.D.	Individual animal data			Average ±S.D.
		No. of phages analyzed	No. of mutants	MF × 10^6	MF × 10^6	No. of phages analyzed	No. of mutants	MF × 10^6	MF × 10^6
Liver	Control (olive oil)	463000	36	77.8		1547000	31	20.0	
		173000	12	69.4		602000	20	33.2	
		1899000	247	130.1		1618000	47	29.0	
		396000	30	75.8	88.2 ± 24.3	1398000	22	15.7	24.5 ± 7.0
	1,10-DAC	391000	77	196.9		1299000	90	69.3	
		1126000	127	112.8		989000	40	40.4	
		371500	63	169.6		1805000	72	39.9	
		244000	36	147.5	156.7 ± 30.8*	1095000	23	21.0	42.7 ± 17.3
	4,10-DAC	192000	72	375.0		731000	79	108.1	
		355500	193	542.9		1446000	182	125.9	
		506500	225	444.2		2080000	233	112.0	
		503000	308	612.3	493.6 ± 90.9**	1751000	311	177.6	130.9 ± 27.8**
	Chrysene	452500	98	216.6		1025000	51	49.8	
		322500	88	272.9		1220000	81	66.4	
		362500	88	242.8		1343000	68	50.6	
		522000	89	170.5	225.7 ± 37.6**	1799000	60	33.4	50.0 ± 11.7*
Spleen	Control (olive oil)	866000	47	54.3		966000	14	14.5	
		1145000	59	51.5		1180000	25	21.2	
		870000	36	41.4		1024000	34	33.2	
		653500	23	35.2	45.6 ± 7.7	1039000	15	14.4	20.8 ± 7.7
	1,10-DAC	1700500	150	88.2		1550000	70	45.2	
		769000	77	100.1		909000	59	64.9	
		1252000	125	99.8		2119000	129	60.9	
		1316000	131	99.5	96.9 ± 5.0**	1837000	110	59.9	57.7 ± 7.5**
	4,10-DAC	469500	109	232.2		550000	56	101.8	
		1506000	525	348.6		1754000	260	148.2	
		1410000	374	265.2		2071000	268	129.4	
		1115000	271	243.0	272.3 ± 45.7**	1438000	219	152.3	132.9 ± 19.9**
	Chrysene	2225000	339	152.4		470500	12	25.5	
		638000	66	103.4		970000	72	74.2	
		1433500	202	140.9		2313000	102	44.1	
		247500	34	137.4	133.5 ± 18.2**	612000	13	21.2	41.3 ± 20.9
Lung	Control (olive oil)	310000	10	32.3		376000	14	37.2	
		296500	28	94.4		263500	11	41.7	
		646000	63	97.5		2016000	73	36.2	
		1402000	73	52.1	69.1 ± 27.8	1429000	23	16.1	32.8 ± 9.9
	1,10-DAC	1911000	539	282.1		1855000	255	137.5	
		1691000	342	202.2		1921000	151	78.6	
		1242000	430	346.2		1807000	183	101.3	
		1826500	652	357.0	296.9 ± 61.7**	2152000	281	130.6	112.0 ± 23.6**
	4,10-DAC	979500	1402	1431.3		1112000	530	476.6	
		1489000	1659	1114.2		1747000	771	441.3	
		1497000	2343	1565.1		1900000	1093	575.3	

Table 1 (*Continued*)

Tissue	Treatment	*lacZ* assay				*cII* assay			
		Individual animal data			Average ± S.D.	Individual animal data			Average ± S.D.
		No. of phages analyzed	No. of mutants	MF × 10^6	MF × 10^6	No. of phages analyzed	No. of mutants	MF × 10^6	MF × 10^6
		1673000	1702	1017.3	1282.0 ± 224.0**	1847000	1005	544.1	509.3 ± 53.0**
	Chrysene	1033000	121	117.1		1577000	61	38.7	
		1526000	371	243.1		2067000	231	111.8	
		1101000	241	218.9		1566000	115	73.4	
		871000	177	203.2	195.6 ± 47.5**	1254000	69	55.0	69.7 ± 27.2
Kidney	Control (olive oil)	447000	36	80.5		546000	21	38.5	
		741500	61	82.3		925000	23	24.9	
		1726000	62	35.9		2183000	70	32.1	
		1948000	72	37.0	58.9 ± 22.5	2244000	57	25.4	30.2 ± 5.6
	1,10-DAC	1609000	214	133.0		2570000	109	42.4	
		1510000	138	91.4		2278000	127	55.8	
		1410000	114	80.9		2621000	96	36.6	
		945000	119	125.9	107.8 ± 22.1*	1658000	67	40.4	43.8 ± 7.2*
	4,10-DAC	1378000	175	127.0		1695500	115	67.8	
		2101000	412	196.1		2526000	270	106.9	
		1447000	235	162.4		2217000	153	69.0	
		1355000	402	296.7	195.5 ± 63.3*	2014000	232	115.2	89.7 ± 21.5**
	Chrysene	1177000	161	136.8		2304000	101	43.8	
		1583000	239	151.0		2415000	157	65.0	
		1187500	241	202.9		2224000	115	51.7	
		1785000	257	144.0	158.7 ± 26.0**	2968000	147	49.5	52.5 ± 7.8**
Bone marrow	Control (olive oil)	421000	20	47.5		580000	12	20.7	
		1034000	48	46.4		1094000	25	22.9	
		1080000	61	56.5		1202000	38	31.6	
		1352000	48	35.5	46.5 ± 7.4	906000	17	18.8	23.5 ± 4.9
	1,10-DAC	1131000	78	69.0		1406000	57	40.5	
		764500	86	112.5		916000	39	42.6	
		1209000	108	89.3		1525000	58	38.0	
		732000	72	98.4	92.3 ± 15.8**	858000	48	55.9	44.3 ± 6.9**
	4,10-DAC	503500	147	292.0		779000	90	115.5	
		621000	239	384.9		1074000	191	177.8	
		1010000	200	198.0		1075000	104	96.7	
		829000	196	236.4	277.8 ± 70.2*	945000	124	131.2	130.3 ± 30.0**
	Chrysene	757500	65	85.8		1179000	20	17.0	
		819000	55	67.2		1046000	46	44.0	
		799500	49	61.3		1331000	33	24.8	
		853500	60	70.3	71.1 ± 9.1*	1392000	25	18.0	25.9 ± 10.9
Colon	Control (olive oil)	369500	17	46.0		1058000	43	40.6	
		1064000	77	72.4		1052000	40	38.0	
		660000	63	95.5		840000	36	42.9	
		676000	55	81.4	73.8 ± 18.0	1187000	33	27.8	37.3 ± 5.8
	1,10-DAC	306500	41	133.8		1715000	85	49.6	
		207000	25	120.8		1250000	57	45.6	

Table 1 (*Continued*)

Tissue	Treatment	*lacZ* assay				*cII* assay			
		Individual animal data			Average ± S.D.	Individual animal data			Average ± S.D.
		No. of phages analyzed	No. of mutants	MF × 10^6	MF × 10^6	No. of phages analyzed	No. of mutants	MF × 10^6	MF × 10^6
		988000	91	92.1		1396000	74	53.0	
		577000	75	130.0	119.2 ± 16.3*	951000	62	65.2	53.3 ± 7.3*
	4,10-DAC	469000	42	89.6		734000	48	65.4	
		369500	211	571.0		1425000	292	204.9	
		1004000	245	244.0		1089000	152	139.6	
		395000	188	475.9	345.1 ± 189.5	954000	187	196.0	151.5 ± 55.7*
	Chrysene	266500	29	108.8		980000	44	44.9	
		379000	52	137.2		1034000	68	65.8	
		1223000	222	181.5		1612000	78	48.4	
		615000	93	151.2	144.7 ± 26.2**	1178000	44	37.4	49.1 ± 10.4

Significantly different from the control group.
* $P<0.05$.
** $P<0.01$.

solution was absorbed to *E. coli* C (*lac*$^-$ *galE*$^-$) at room temperature for 20–30 min. For titration, appropriately diluted phage–*E. coli* solution was mixed with LB top agar (containing 10 mM $MgSO_4$) and plated onto dishes containing bottom agar. The remaining phage–*E. coli* solution was mixed with LB top agar containing phenyl-β-D-galactoside (3 mg/mL) and plated as described above. The mutant frequency (MF) was calculated by the following formula:

mutant frequency = (total number of plaques on selection plates/total number of plaques on titer plates) × dilution factor.

The significance of differences in the mutant frequency between the treated and control groups was analyzed by using Student's *t*-test and Welch's *t*-test in combination with the *F*-test.

2.2.4.2. cII mutant frequency determination. In the present study, we examined the mutagenicity in the lambda *cII* gene, which is also integrated as a lambda vector gene, which serves as another selective marker as reported previously in the *lacI* transgenic BigBlue mouse [24]. The positive selection for *cII* mutants was performed according to the method of Jakubczak et al. [24] with a slight modification as previously reported [9]. Briefly, the phage solution was absorbed to *E. coli* G1225 (*hfl*$^-$) at room temperature for 20–30 min. For titration, appropriately diluted phage–*E. coli* solution was mixed with LB top agar (containing 10 mM $MgSO_4$) and plated onto dishes containing bottom agar and the plates were incubated at 37 °C for 24 h. The remaining phage–*E. coli* solution was mixed with LB top agar and plated onto dishes containing bottom agar. The plates were incubated at 25 °C for 48 h for selection of *cII* mutants. The wild-type phage, recovered from Muta^{TM}Mice, has a *cI*$^-$ phenotype, which permits plaque formation with the *hfl*$^-$ strain at 37 °C but not at 25 °C. The mutant frequency was calculated by the following formula:

mutant frequency = (total number of plaques on selection plates/total number of plaques on titer plates) × dilution factor.

The significance of differences in the mutant frequency between the treated and control groups was analyzed by using Student's *t*-test and Welch's *t*-test in combination with the *F*-test.

2.2.5. Sequencing of mutants

The entire lambda *cII* region was amplified directly from mutant plaques by Taq DNA polymerase (Takara Shuzo, Tokyo, Japan) with primers P1, 5′-AAAAAGGGCATCAAATTAAACC-3′ and P2, 5′-CCGAAGTTGAGTATTTTTGCTGT-3′ as previously reported [9] (Fig. 2). A 446 bp PCR product was puri-

fied with a microspin column (Amersham Pharmacia, Tokyo, Japan) and then used for a sequencing reaction with the Ampli Taq cycle sequencing kit (PE Biosystems, Tokyo, Japan) using the primer P1. The reaction product was isolated by ethanol precipitation and analyzed with the ABI PRISM™ 310 genetic analyzer (PE Biosystems). In this study, about 40 mutants were subjected to sequence analysis in each group both in the liver and lung.

3. Results

3.1. Mutant frequencies by 1,10-DAC, 4,10-DAC and chrysene

Chrysene and its diaza-analogs, 1,10-DAC and 4,10-DAC, were tested for in vivo mutagenicity using *lacZ* transgenic mice (Muta™Mice). Chrysene and 4,10-DAC were injected at the total dose of 800 mg/kg

Table 2
Sequences of *cII* mutations in the liver of 1,10-DAC-treated Muta™Mouse

Mutant no.	Position	Mutation	Sequence			Amino acid change
C1	117	G to T	TCG	CAG	ATC	Gln to His
C2	42	G to T	ATC	GAG	AGT	Glu to Asp
C3	126	G to T	AGC	AGG	TGG	Arg to Ser
C4	40–43	−GA	GAG	AGT	GCG	Frameshift
C5	166	G to T	CTT	GCT	GTT	Ala to Ser
C6	233	T to A	ATT	CTC	ACC	Leu to His
C7	132	G to T	TGG	AAG	AGG	Lys to Asn
C8	89	C to T	ACA	GCG	GAA	Ala to Val
C9	11	C to TA	CGT	GCA	AAC	Frameshift
C10	178	T to A	GAA	TGG	GGG	Trp to Arg
C11	197	A to G	GAC	GAC	ATG	Asp to Gly
C12	179	G to T	GAA	TGG	GGG	Trp to Leu
C13	29	C to A	GAG	GCT	CTA	Ala to Asp
C14	101	G to T	GTG	GGC	GTT	Gly to Val
C15	89	C to T	ACA	GCG	GAA	Ala to Val
C16	117	G to C	TCG	CAG	ATC	Gln to His
C17	40	G to A	ATC	GAG	AGT	Glu to Lys
C18	294	A to C	TTC	TGA	–	Stop to Cys
C19	150	G to T	CCA	AAG	TTC	Lys to Asn
C20[a]	294	A to C	TTC	TGA	–	Stop to Cys
C21	211	G to A	TTG	GCG	CGA	Ala to Thr
C22	111	G to T	GAT	AAG	TCG	Lys to Asn
C23	123	C to A	ATC	AGC	AGG	Ser to Arg
C24	160	C to A	ATG	CTG	CTT	Leu to Met
C25	173	T to C	GTT	CTT	GAA	Leu to Pro
C26	125	G to C	AGC	AGG	TGG	Arg to Thr
C27	−3	C to G	tta	cat	ATG	Base substitution in the 5′-flanking region
C28	29	C to A	GAG	GCT	CTA	Ala to Asp
C29[a]	111	G to T	GAT	AAG	TCG	Lys to Asn
C30	38	T to A	CGA	ATC	GAG	Ile to Asn
C31	40	G to T	ATC	GAG	AGT	Glu to Stop
C32	163	C to T	CTG	CTT	GCT	Leu to Phe
C33	79	G to T	ACT	GAG	AAG	Glu to Stop
C34	57	C to A	CTT	AAC	AAA	Asn to Lys
C35	89	C to T	ACA	GCG	GAA	Ala to Val
C36	179–184	−G	TGG	GGG	GTC	Frameshift
C37	113	C to A	AAG	TCG	CAG	Ser to Stop
C38	89	C to G	ACA	GCG	GAA	Ala to Gly
C39	123	C to G	ATC	AGC	AGG	Ser to Arg
C40	125	G to A	AGC	AGG	TGG	Arg to Lys

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

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 Muta™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 Glu
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[a]	113	C to T	AAG	TCG	CAG	Ser to Leu
D11	42	G to T	ATC	GAG	AGT	Glu 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	Glu 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[a]	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

[a] 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 frequencies 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 Muta™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 Muta™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 Muta™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 *cII* mutations in the lung of 1,10-DAC-treated Muta™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 Muta™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[a]	115	C to A	TCG	CAG	ATC	Gln to Lys
d21[a]	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[a]	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

[a] 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 Muta™Mouse. One of the authors (T.S.) reported that the median toxic dose (TD_{50}) 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 Muta™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 Gry
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 (%) = *lacZ* MF obtained by test chemical/spontaneous *lacZ* MF × 100.
Mutagenic activity = fold-increase in *lacZ* MF/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