Anticancer activities of alkylating pyrrole–imidazole polyamides with specific sequence recognition

Ken-Ichi Shinohara[a], Toshikazu Bando[a] and Hiroshi Sugiyama[a,b]

In recent years, many diseases including cancer and hereditary and viral diseases have been understood at the DNA sequence level. Direct control of the expression level of a specific gene would provide a promising approach for knowledge-based therapy. *N*-methylpyrrole and *N*-methylimidazole polyamides are a new type of small compound that precisely bind to the minor groove of the DNA duplex in a sequence-specific fashion and recruit alkylating agents to the target sequence. We designed and synthesized a series of sequence-specific alkylating Py–Im polyamide conjugates that selectively alkylate predetermined DNA sequences. We have shown that sequence-specific alkylating agents possess gene-silencing activities when they alkylate coding regions of template strands and show promising potency against human cancer cell lines and xenografts possessing human cancer cells. In this study, we focus on recent progress in alkylating Py–Im polyamides with regard to sequence specificity and biological activities, and the future direction of the rational molecular design of genetic switches in the post-genome era is described. *Anti-Cancer Drugs* 21:228–242 © 2010 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

The completion of the Human Genome Project provides a new grand challenge for the broader research community. Deciphering the information encoded in the functional genome, including thousands of predicted gene products, will require a variety of new scientific tools and methods. Recently, small molecules have emerged in starring roles as tools for determining macromolecular structures, probing molecular interaction and designing drugs [1–4]. These interests reflect a fascination with research at the interface of chemistry and biology known as 'chemical biology'. Cell-permeable small molecules that bind to DNA and perturb the functions of genes can be particularly useful tools in studies that require temporal or spatial control over the gene target. In addition, small molecules that recognize specific DNA sequences might uncover novel therapeutic targets for human disease, and might serve as templates for therapeutic design. Today, cancer is one of the most serious diseases known to humankind because one-third of individuals die from this genomic disease in advanced nations. Many challenges such as genome-based drug development and cancer chemotherapy tailored to individual genomic construction have attracted considerable attention [5]. In addition, DNA-alkylating and cleaving agents have been used as antitumor agents. Their selectivity to cancer cells usually depends on the rapid proliferation of cancer cells compared with normal cells; however, severe side effects are caused by the nonspecific DNA alkylation of normal cells [6].

One important question to consider is whether the introduction of sequence selectivity to DNA-targeting agents can improve their efficacy as anticancer agents. To address this question, we have designed and synthesized various types of sequence-specific DNA-alkylating agents by the conjugation of *N*-methylpyrrole (Py)–*N*-methylimidazole (Im) polyamides with DNA-alkylating agents. The purpose of this study is to focus on what biological activities are induced in living cells by sequence-specific DNA-alkylating agents. We expect that such progress in molecular design and the functional analysis of sequence-specific DNA-alkylating agents steadily approaches the goal of developing tailor-made anticancer agents.

Sequence recognition by pyrrole–imidazole polyamides

DNA stores the biological information required for life. The four bases, adenine (A), thymine (T), guanine (G) and cytosine (C), contain information that is communicated by the two processes of transcription and translation. Chemical substances that can interfere with these four bases severely affect cellular functions in all organisms. Such substances are very important to molecular recognition and medicine. Small molecules that bind to minor grooves in DNA by recognizing a specific DNA sequence have been extensively examined for biological activity such as antitumor and antibacterial activities [7]. For

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example, antitumor antibiotics, distamycin A and netropsin, contain a common 3-Py and 2-Py skeleton, respectively, and are representative of classical minor groove binders that recognize consecutive A–T base pairs in DNA [8].

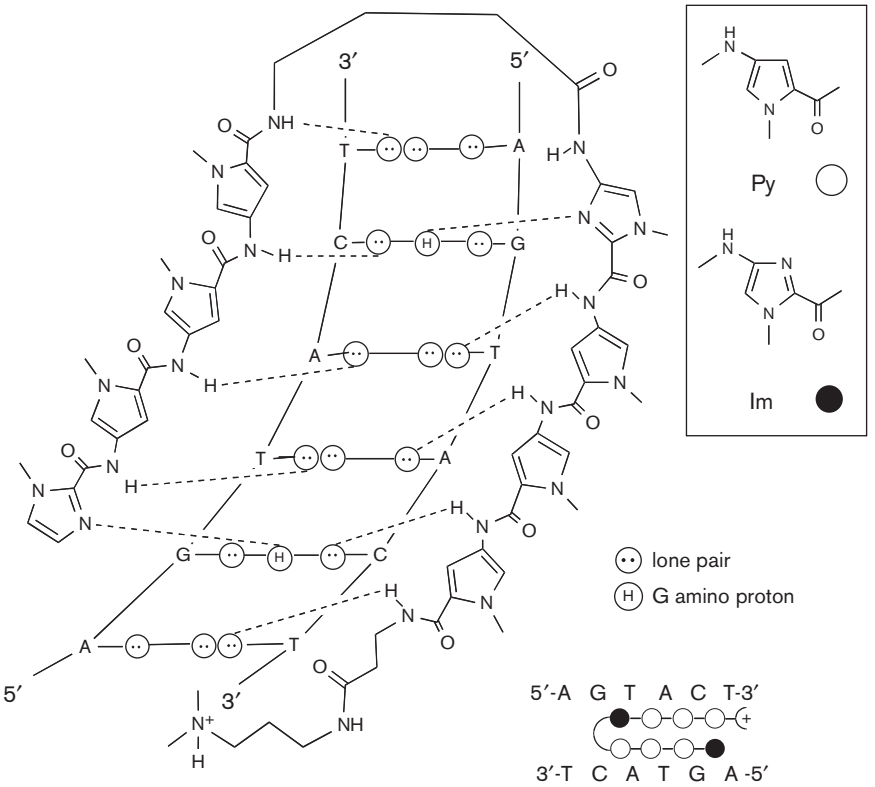
Dickerson *et al.* [9] solved the crystal structure of a 1 : 1 complex of netropsin and d(CGCGAATTCGCG), in which netropsin binds to the minor groove of the middle of the 5′-AATT-3′ sequence. On the basis of these contributions to the concept of sequence-specific recognition in the minor groove, Dervan *et al.* showed that Py–Im polyamides were minor groove-binding molecules that precisely recognize each of the four Watson–Crick basepairs, as shown in Fig. 1. An antiparallel pairing of Im opposite Py (Im/Py) recognizes a G–C base pair, whereas a Py/Py pair recognizes A–T or T–A base pairs [10–13].

Py–Im hairpin polyamides with γ-aminobutyric acid turn were shown to be useful DNA-binding units, with increased specificity and affinity, as confirmed by nuclear magnetic resonance (NMR) spectroscopy [14]. Hairpin polyamides have advantages such as ease and flexibility of design, automation-driven solid-phase synthesis, cell permeability because of their low molecular weight, the presence of flexible sites for covalent attachment to other molecules, and use in analytical applications [15,16]. The binding constants and sequence specificity of Py–Im hairpin polyamides are comparable with those of transcription factors [8,12]. Thus, the expression of various genes has been silenced by competitive binding of Py–Im hairpin polyamides to their regulatory sequences [17].

Cooperative alkylation by duocarmycin A and distamycin A

DNA-alkylating agents such as the nitrosoureas, mitomycin C and cisplatin, which constitute a major class of antitumor drugs, have long been of interest for their biological properties, and are routinely used for cancer therapy. These drugs are relatively toxic to normal cells. One important question is whether the introduction of sequence selectivity to an alkylating agent can improve its efficacy as an anticancer agent. In addition, the question arises as to whether one can tailor the binding preference of DNA-binding agents to particular sequences and thereby create a tailor-made antitumor agent. Progress in our study of DNA alkylation has led to methods for developing novel antitumor agents with sequence recognition ability. DNA sequence specificity is an important component contributing to the cytotoxic potency of several alkylating Py–Im polyamides [18].

Fig. 1



Binding model of pyrrole (Py)–imidazole (Im) polyamides based on the recognition rule in the minor groove.

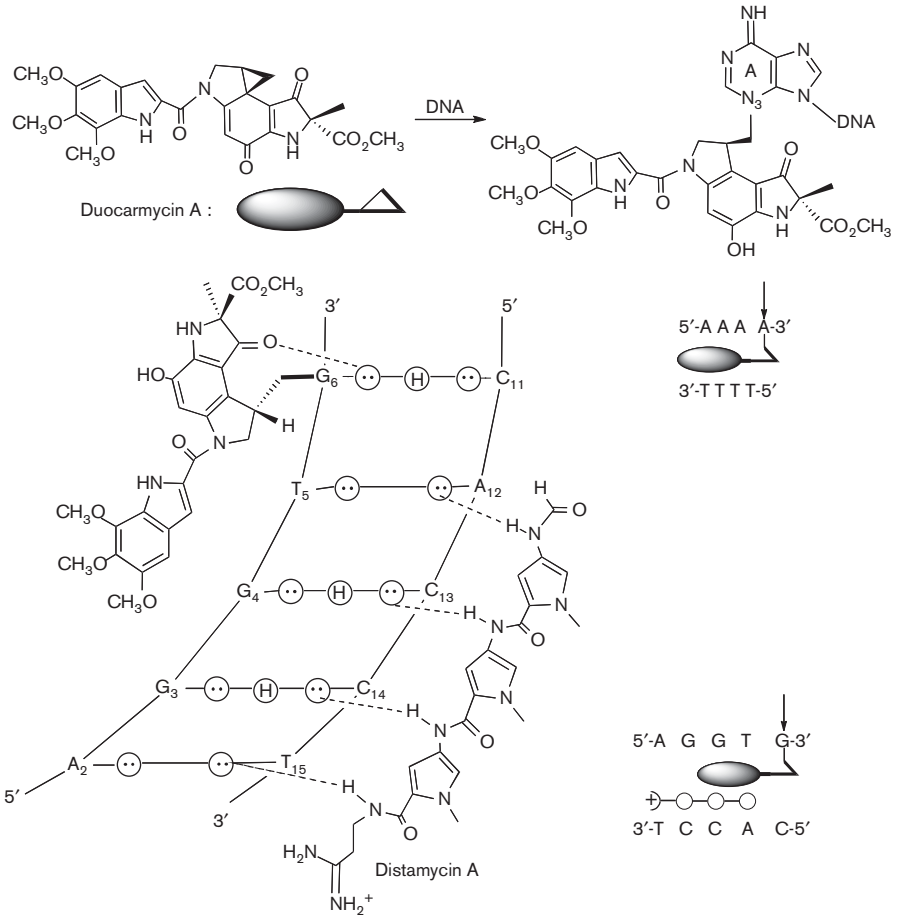
To develop a sequence-specific DNA-alkylating agent, we chose to use the sequence-recognition ability of Py–Im polyamides and the alkylating moieties of antitumor antibiotics [19]. We were especially attracted by duocarmycin A, a minor groove-binding antitumor antibiotic produced by *Streptomyces* species that alkylates adenine N3 at the 3′ end of sequences of three or more consecutive A–T base pairs in DNA [20] (Fig. 2). Almost a decade ago, we discovered that the addition of distamycin A markedly modulates alkylation sites, primarily at G residues in GC-rich sequences, by forming a cooperative heterodimer between duocarmycin A and distamycin A [21]. The NMR-refined structure of a duocarmycin A–distamycin A–d(CAGGTGGT)/d(ACCA CCTG) complex showed that heterodimers of duocarmycin A and distamycin A tightly bind to the minor groove of DNA duplexes. Importantly, the replacement of distamycin A with various Py–Im triamides changes the sequence-specific alkylation by duocarmycin A in a predictive manner [22], with two Py units of distamycin A recognizing the complementary strand of the reacting octamer according to the basepair recognition rule of Py–Im polyamides in the minor groove. These results suggest that Py–Im polyamides can be used as versatile sequence-recognition components of sequence-specific DNA-alkylating conjugates.

Design and biological properties of DNA-alkylating agents

To develop a sequence-specific DNA-alkylating agent, we used the sequence-recognition ability of Py–Im polyamides and the various types of DNA-alkylating moiety. Sequence-specific DNA-alkylating activities were generally evaluated by thermally induced DNA strand cleavage with high-resolution denaturing polyacrylamide gel electrophoresis (PAGE).

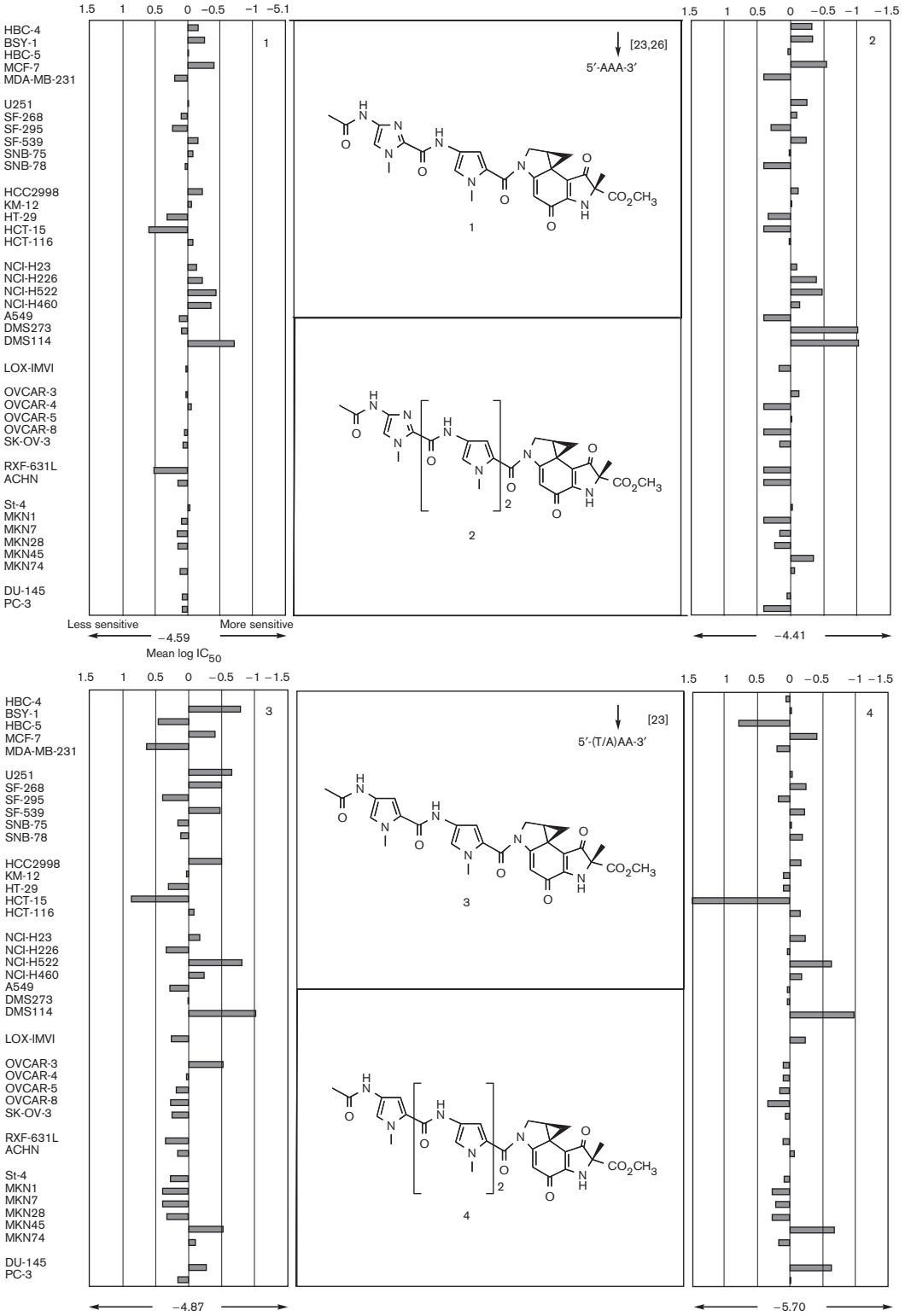
We first designed and synthesized Py–Im duocarmycin A conjugates 1–4 and investigated their sequence-specific alkylating activities [23]. Conjugates 1 and 3 selectively alkylated the 3′ end of A in AT-rich sequences, although conjugate 3 is much more reactive toward double-strand

Fig. 2



The chemical structure of duocarmycin A and distamycin A. Schematic representations of adenine N3-alkylation in an AT-rich sequence by duocarmycin A and alkylation by heterodimer formation between duocarmycin A and distamycin A. The arrow indicates the site of alkylation.

Fig. 3



Chemical structures of pyrrole–imidazole duocarmycin A conjugates (1–4) and graphs for each conjugate showing the mean of 50% growth inhibition (IC_{50}) against a panel of 39 human cancer cell lines. Columns extending to the right indicate more sensitive to agents; columns extending to the left indicate less sensitive to conjugates. One unit represents one logarithmic difference [24].

DNA fragments than is conjugate 1, judging from PAGE analysis. However, the efficiency of DNA alkylation by the conjugates was relatively low compared with that of duocarmycin A alone. In the presence of distamycin A, alkylation by conjugates 1 and 3 predominantly occurred at the 3′ end of G in 5′-GTG-3′ and 5′-(T/A)TG-3′ sequences, respectively. The cytotoxicities of conjugates 1–4 against 39 human cancer cell lines [24] are shown in Fig. 3. The average of 50% growth-inhibiting concentrations (IC_{50}) of conjugates 1–4 was moderate ($-5.70 < \log IC_{50} < -4.41$, Fig. 3), and each correlation coefficient between conjugates 1 and 4 was relatively moderate (0.50–0.73), as shown in Table 1.

It was assumed that the chemical instability of duocarmycin A in neutral pH resulted in low antitumor activity. Therefore, we applied cyclopropylpyrroloindole (CPI) [25] as an alternative DNA-alkylating moiety, which is a more stable derivative of duocarmycin A. In PAGE analysis, Py–Im CPI conjugate 5 alkylated both the 3′ end of A in AT-rich sequences and the 3′ end of Pu in 5′-PyGA CPu-3′/5′-PyGTCPu-3′ through cooperative homodimer formation [26]. These results indicated that the substitution of the alkylating moiety with CPI increased the planarity of the molecules, which allow sequence-specific double alkylation of dsDNA. The average log IC_{50} for conjugates 5–8 indicated that they had 10-fold higher cytotoxic effects on 39 human cancer cell lines than conjugates 1–4 ($-6.58 < \log IC_{50} < -5.65$, Fig. 4). Each correlation coefficient between conjugates 5 and 8 was relatively high (0.69–0.94), as Py–Im diamide or triamide do not have good sequence-specificity (Table 1).

Therefore, we improved the design of various types of CPI and Py–Im polyamides containing vinyl linkers 9–15, as shown in Figs 5 and 6. Importantly, the insertion of a vinyl linker between CPI and the Py–Im polyamides dramatically enhances DNA-alkylating reactivity. Conjugate 9, which possesses a vinyl linker relative to conjugate 5, strongly alkylated each 3′ end of Pu in 5′-PyGACPu-3′/5′-PyGTCPu-3′ through the cooperative homodimer formation [27]. In particular, the molecular design of sequence-specific interstrand cross-linking agents by Py–Im conjugates was achieved by using a vinyl linker [28,29]. These results confirmed that incorporation of a vinyl linker substantially improved the DNA-alkylating activity of Py–Im CPI conjugates. Therefore, the cytotoxicity for conjugates 9–15 against human cancer cell lines by more than 100-fold compared with conjugates 5–8, which contain no vinyl linkers ($-8.26 < \log IC_{50} < -7.20$, Figs 5 and 6).

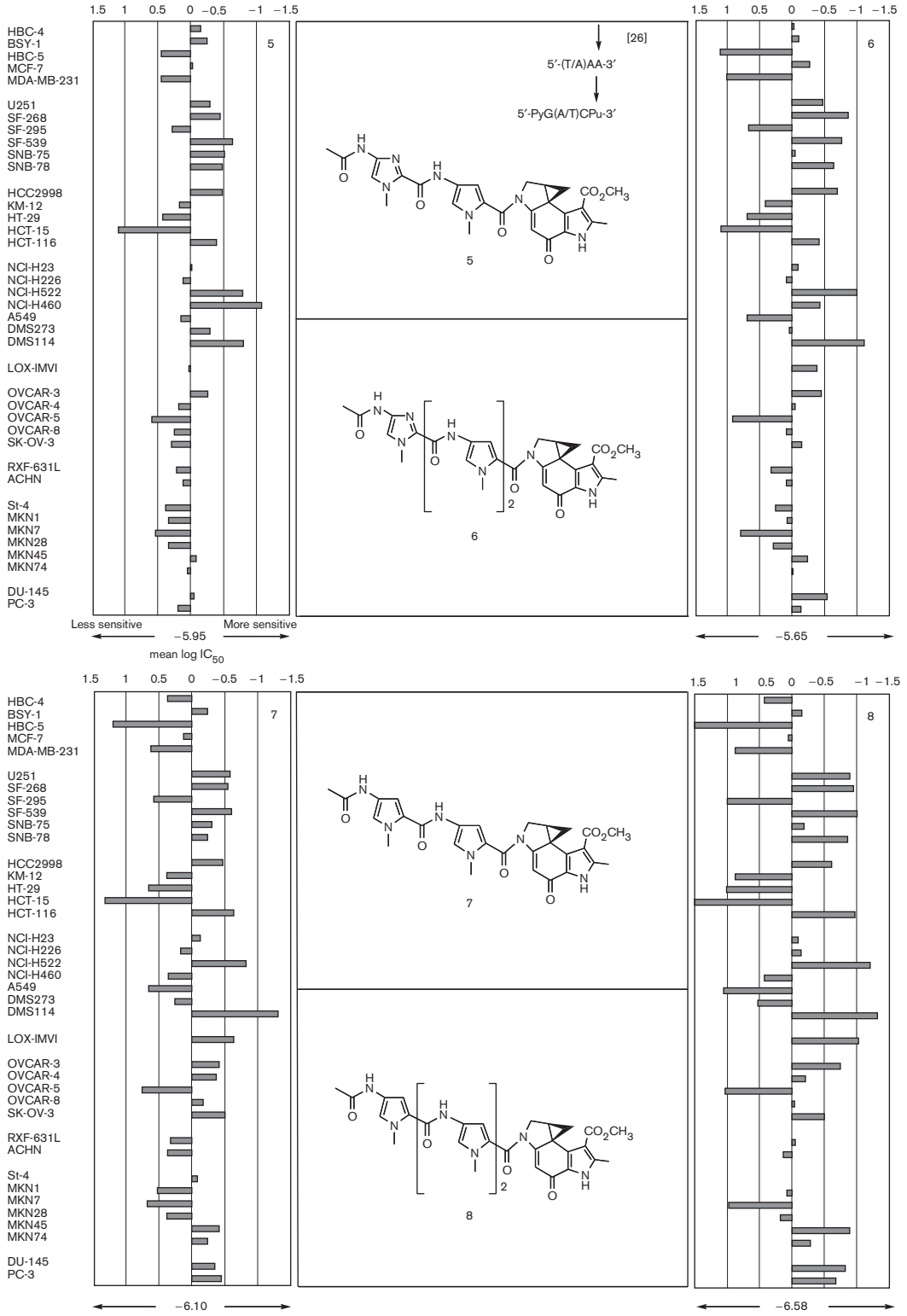
Each correlation coefficient around conjugates 9 to 15 was still high (0.65–0.95), as shown in Table 1. The sequence specificities of Py–Im polyamides were not reflected on correlation coefficients, as these conjugates confer a high

Table 1 The correlation coefficient among conjugates 1–26

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
2	0.69																								
3	0.71	0.69																							
4	0.65	0.50	0.73																						
5	0.68	0.55	0.76	0.71																					
6	0.61	0.49	0.80	0.74	0.84																				
7	0.55	0.41	0.74	0.73	0.69	0.89																			
8	0.46	0.31	0.68	0.78	0.67	0.90	0.94																		
9	0.55	0.51	0.72	0.79	0.68	0.85	0.87	0.88																	
10	0.52	0.49	0.66	0.77	0.66	0.82	0.83	0.87	0.94																
11	0.46	0.45	0.61	0.67	0.63	0.74	0.70	0.74	0.90	0.86															
12	0.40	0.44	0.51	0.61	0.56	0.64	0.56	0.61	0.80	0.85	0.86														
13	0.52	0.44	0.69	0.74	0.70	0.89	0.92	0.93	0.93	0.91	0.81	0.71													
14	0.46	0.28	0.65	0.76	0.59	0.79	0.85	0.91	0.87	0.86	0.77	0.65	0.87												
15	0.56	0.52	0.69	0.76	0.68	0.87	0.90	0.90	0.93	0.94	0.80	0.73	0.95	0.83											
16	0.59	0.51	0.55	0.46	0.64	0.56	0.38	0.36	0.55	0.54	0.58	0.56	0.47	0.36	0.51										
17	0.49	0.56	0.46	0.62	0.60	0.50	0.36	0.40	0.62	0.58	0.63	0.65	0.52	0.42	0.55	0.61									
18	0.29	0.34	0.38	0.58	0.51	0.46	0.45	0.49	0.63	0.71	0.69	0.74	0.53	0.53	0.60	0.49	0.65								
19	0.46	0.50	0.46	0.59	0.52	0.49	0.39	0.39	0.62	0.62	0.59	0.59	0.51	0.43	0.62	0.63	0.64	0.60							
20	0.50	0.53	0.37	0.49	0.52	0.46	0.33	0.37	0.59	0.55	0.58	0.58	0.48	0.35	0.51	0.56	0.88	0.55	0.57						
21	0.49	0.53	0.55	0.63	0.62	0.59	0.47	0.54	0.74	0.74	0.74	0.74	0.68	0.58	0.66	0.68	0.85	0.64	0.63	0.77					
22	0.32	0.43	0.42	0.60	0.56	0.48	0.35	0.46	0.67	0.67	0.72	0.75	0.54	0.51	0.55	0.64	0.83	0.69	0.66	0.74	0.90				
23	0.52	0.49	0.44	0.70	0.64	0.55	0.42	0.49	0.72	0.71	0.75	0.80	0.60	0.54	0.63	0.67	0.79	0.71	0.79	0.71	0.83	0.86			
24	0.65	0.54	0.64	0.75	0.75	0.81	0.71	0.75	0.85	0.84	0.79	0.71	0.84	0.71	0.82	0.65	0.60	0.56	0.69	0.55	0.74	0.67	0.80		
25	0.28	0.32	0.28	0.52	0.46	0.45	0.38	0.44	0.62	0.60	0.64	0.72	0.52	0.44	0.55	0.53	0.66	0.58	0.71	0.62	0.69	0.74	0.79	0.61	
26	0.38	0.43	0.33	0.51	0.55	0.45	0.36	0.36	0.58	0.48	0.61	0.61	0.47	0.29	0.45	0.55	0.76	0.53	0.59	0.77	0.71	0.73	0.75	0.53	0.86

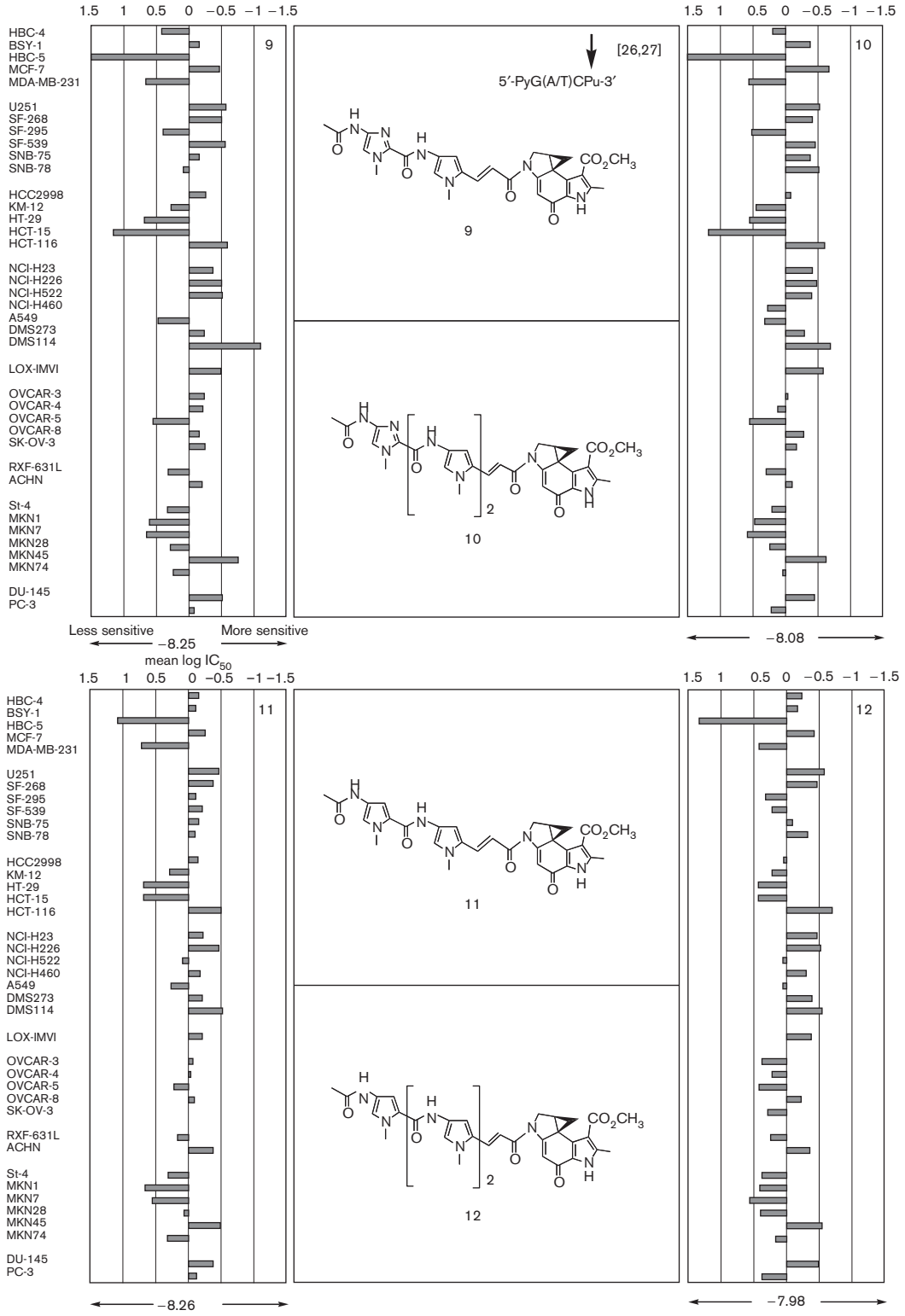
In general, the panel patterns of drugs possessing a common mechanism resembled one another ($r > 0.75$).

Fig. 4



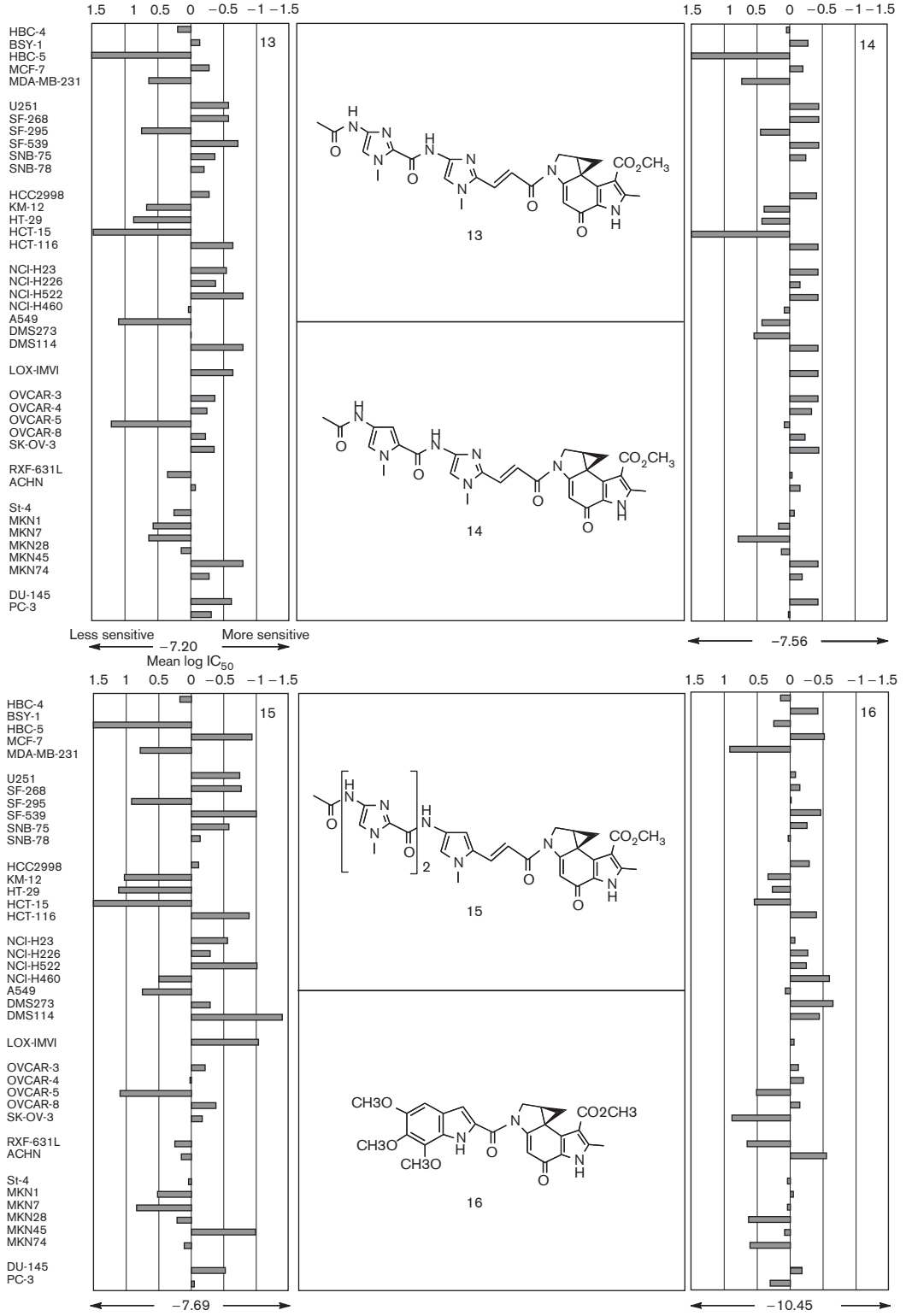
Chemical structures of pyrrole–imidazole cyclopropylpyrroloindole conjugates (5–8) and graphs for each conjugate showing the mean of 50% growth inhibition (IC_{50}) against a panel of 39 human cancer cell lines. Columns extending to the right indicate more sensitive to agents; columns extending to the left indicate less sensitive to conjugates. One unit represents one logarithmic difference.

Fig. 5



Chemical structures of pyrrole–imidazole cyclopropylpyrroloindole conjugates with vinyl linker (9–12) and graphs for each conjugate showing the mean of 50% growth inhibition (IC_{50}) against a panel of 39 human cancer cell lines. Columns extending to the right indicate more sensitive to agents; columns extending to the left indicate less sensitive to conjugates. One unit represents one logarithmic difference.

Fig. 6



Chemical structures of pyrrole–imidazole cyclopropylpyrroloindole conjugates with vinyl linker (13–15) and DU-86 (16): graphs for each conjugate showing the mean of 50% growth inhibition (IC_{50}) against a panel of 39 human cancer cell lines. Columns extending to the right indicate more sensitive to agents; columns extending to the left indicate less sensitive to conjugates. One unit represents one logarithmic difference.

cytotoxicity by the double alkylation to dsDNA. Notably, we examined in detail comparative studies of DNA sequence-specific alkylation and the antitumor activity of the alkylating Py–Im conjugates 1, 5 and 9 [26]. Importantly, the DNA-alkylating efficiency of conjugate 9 with vinyl linkers was much higher than that of conjugates 1 and 5 using DNA fragments. The double alkylation of the DNA oligonucleotides containing target DNA sequence was completed within 5 min under the nanomolar concentration of conjugate 9.

The observation of efficient sequence-specific alkylation by the conjugates encouraged us to further examine the biological activity induced by specific DNA alkylation by these agents. For the accurate sequence recognition, we synthesized Py–Im hairpin polyamides and CPI conjugates with vinyl linkers [30]. Py–Im hairpin polyamides with a γ-aminobutyric acid turn were shown to be useful DNA-binding units, with increased specificity and affinity confirmed by NMR spectroscopy [14]. For example, conjugate 17 alkylates DNA at the purine in 5′-(A/T)GCCPu-3′, and conjugate 18 alkylates DNA at the purine in 5′-(A/T)G(A/T)CPu-3′, at nanomolar concentrations according to the Py–Im recognition rule. Hairpin-type alkylating Py–Im polyamides 17–21 also showed effective DNA alkylation with high sequence-specific recognition ability ($-6.67 < \log IC_{50} < -5.40$, Figs 7 and 8). The mean log IC_{50} values of polyamides 17–21 were roughly equivalent to the values for mitomycin C (–6.0) and cisplatin (–5.2).

In particular, we found that alkylating Py–Im polyamides 17 and 18, which differ only in that the C–H atoms are substituted by an N atom in the second ring, showed significantly different cytotoxicity in the 39 human cancer cell line panel shown in Fig. 7 [31]. The correlation coefficients between conjugates 17 and 18 were relatively moderate (0.65). Each correlation coefficient between conjugates 17 and 21 varied (0.55–0.88). These results suggest that differences in sequence specificity might affect the pattern of cytotoxicities.

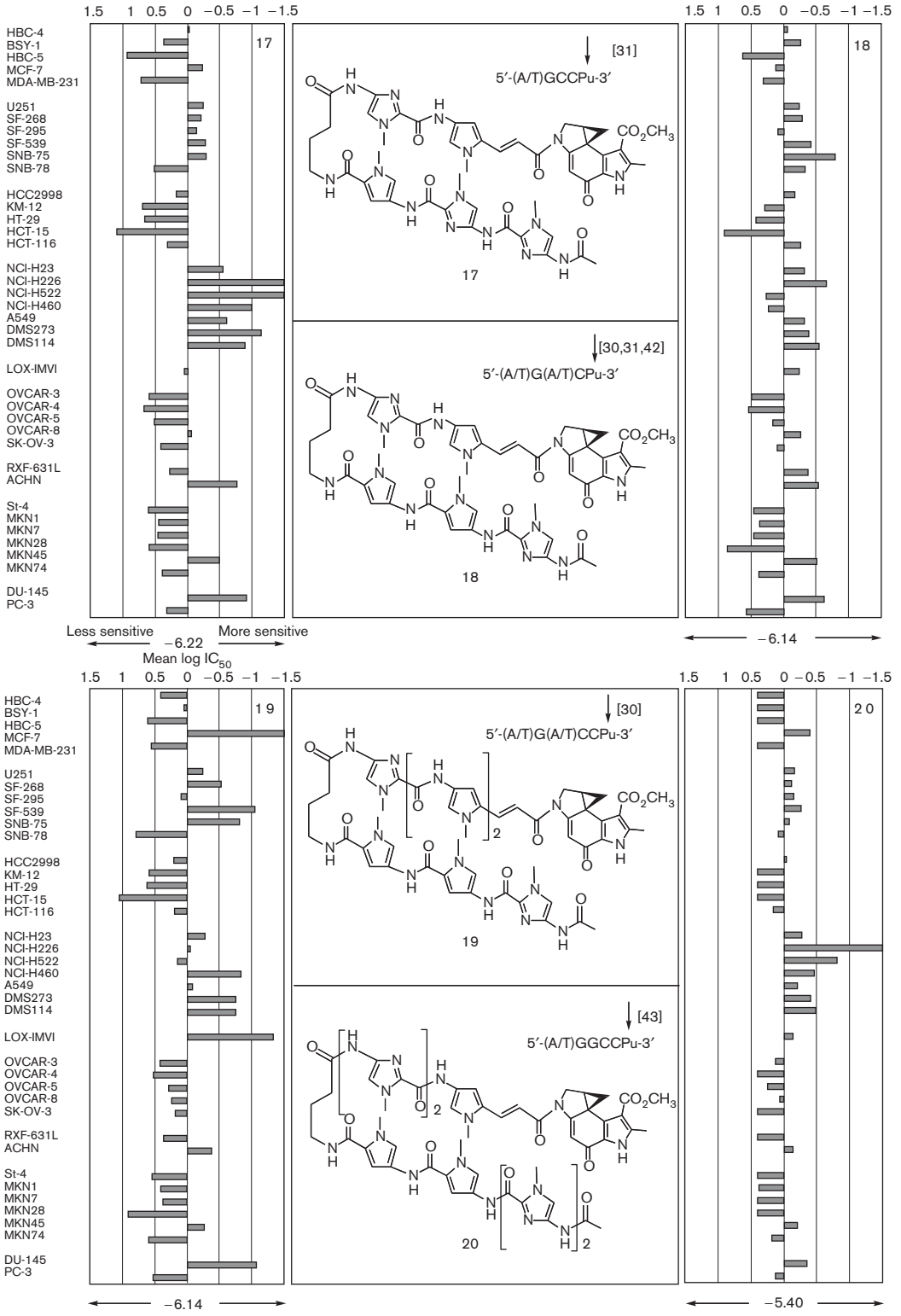
Although highly efficient sequence-specific DNA alkylation at the target sequence of Py–Im polyamides was achieved, the CPI moiety was prepared from duocarmycin A through several chemical transformation steps with a relatively low yield. Therefore, we introduced 1,2,9,9a-tetrahydrocyclopropa[1,2-c]benz[1,2-e]indol-4-one (CBI) or its precursor, *seco*-CBI, as an alkylating moiety [32,33] to prepare CBI conjugate *22* [34]. Conjugate *22* showed sequence-specific alkylating activity corresponding with the CPI conjugate. Interestingly, Py–Im CBI conjugate *22* specifically underwent DNA alkylation at A despite having a similar binding orientation, whereas DNA alkylation by the CPI conjugates occurred both at A and G [35].

Similarly, polyamides with vinyl linker moieties were difficult to synthesize because of their low chemical stability under basic and acidic conditions. Thus, we identified stable substitutes and selected 2-carbonyl-5-aminoindole as a linker [36]. The DNA-alkylating activities of Py–Im–CBI conjugates *23* and *24* clearly indicated that the cyclopropane ring of conjugates with natural configurations (*23*) shows strong DNA-alkylating activity compared with that of conjugates with unnatural configurations (*24*). The antitumor activities of the Py–Im conjugates *21*–*24* were comparable with similar sequence-specific alkylation ($-7.58 < \log IC_{50} < -6.58$, Fig. 8). In particular, each correlation coefficient between conjugates *21* and *23* was high (0.83–0.90). Importantly, the introduction of an indole linker greatly facilitated the synthesis of sequence-specific alkylating Py–Im polyamides because CBI could be synthesized from commercially available starting materials using a general synthetic methodology. These conjugates selectively and efficiently alkylate matching sequences, 5′-(A/T)G(A/T)CCA-3′, of DNA fragments. The reactivities of *seco*-CBI derivatives were equal to those of the corresponding CBI conjugates. These modifications greatly facilitated the synthesis of sequence-specific alkylating Py–Im polyamides by enabling the effective use of solid-phase Py–Im polyamide synthesis. More recently, we designed and synthesized Py–Im CBI conjugates *25* and *26*, which target both strands of the double-stranded region of the human telomere repeat sequences, 5′-d$(TTAGGG)_n$-3′/5′-d$(CCCTAA)_n$-3′ [37]. High-resolution sequencing gel electrophoresis showed that the conjugates selectively recognize and alkylate G- and C-rich sequences at target sites in telomere duplex repeats at nanomolar concentrations *in vitro*. Although examination of the biological effects of these agents is in progress, it is expected that specific damage in the telomeric region would lead to shorting of telomere length, which could show novel antitumor activity.

COMPARE analysis of the specific cytotoxicity of Py–Im polyamide conjugates

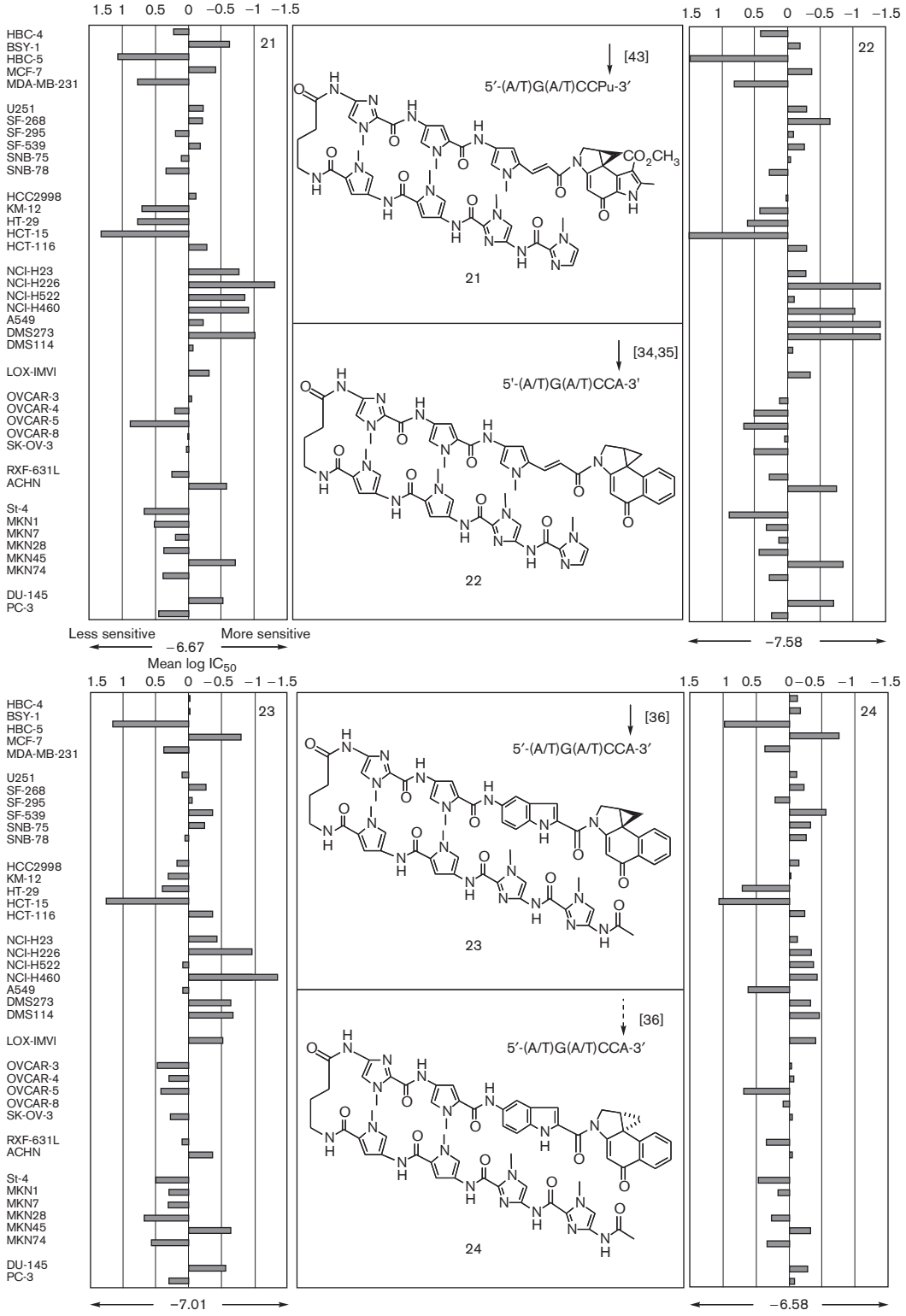
Comparison of the growth inhibition patterns of compounds 1–26 (COMPARE analysis) was considered from the correlation coefficients (r) for the mean IC_{50} values of compounds against all combinations, as shown in Table 1 [24]. A graph of the means showed that the two types of Py–Im conjugates correlated well with each other ($r > 0.75$), confirming the notion that sequence specificity may correlate with cytotoxicity. Importantly, we observed a substantial difference in cytotoxicity between these structural analogue conjugates, despite them having a common DNA-alkylating mechanism (purine N3 alkylation). Interestingly, the COMPARE analysis revealed that the *r* values among 4–15, 17–21 and DU-86 (16) were relatively low, despite these conjugates having the same DNA-alkylating moiety (CPI).

Fig. 7



Chemical structures of hairpin pyrrole–imidazole cyclopropylpyrroloindole conjugates with vinyl linker 17–20 and graphs for each conjugate showing the mean of 50% growth inhibition (IC_{50}) against a panel of 39 human cancer cell lines. Columns extending to the right indicate more sensitive to agents; columns extending to the left indicate less sensitive to conjugates. One unit represents one logarithmic difference.

Fig. 8



Chemical structures of hairpin pyrrole–imidazole conjugates with vinyl-linked cyclopropylpyrroloindole (21), vinyl linked CBI (22), and indole-linked CBI (23, 24): graphs for each conjugate showing the mean of 50% growth inhibition (IC_{50}) against a panel of 39 human cancer cell lines. Columns extending to the right indicate more sensitive to agents; columns extending to the left indicate less sensitive to conjugates. One unit represents one logarithmic difference.

COMPARE analysis of the mean activities against a panel of 39 human cancer cell lines recorded for three different alkylating polyamides ImImPyPyγImPyPy-vinyl-CPI 21, ImImPy-PyγImPyPy-vinyl-CBI 22 and ImImPyPyγImPy-indole-CBI 23, which recognize the same DNA sequences, indicated higher correlation coefficients ($r > 0.83$). It is generally accepted that higher correlation coefficients ($r > 0.75$) in a COMPARE analysis are observed for anticancer agents possessing the same reaction mechanism. Interestingly, some conjugates indicated higher activities against lung cancer cell lines (NCI-H23, NCI-H226, NCI-H522, NCI-H460, A549, DMS273 and DMS114). These results suggest that cancer cells have weak point sequences, like Achilles' heel. Therefore, the ultimate goal of our research is to discover these sequences and synthesize compounds that selectively target these sequences, which would further the development of individual cancer-specific antitumor drugs.

In addition, we carried out COMPARE analysis of conjugates 1–26 with standard antitumor agents (Table 2). The growth inhibition patterns of conjugates 1–26 indicated that they had a higher correlation with SN-38 (a topoisomerase I inhibitor) and doxorubicin (a DNA intercalator) than with cisplatin (a DNA-alkylating agent). It is reasonable that DU-86 (16) shows a higher correlation with melphalan, which has the same alkylating mechanism. However, it is important to note that alkylating polyamides show a higher correlation with typical topoisomerase I inhibitors. The unique antitumor activities of the Py–Im conjugates suggest that they are different from simple DNA-alkylating agents.

Table 2 The results of COMPARE analysis of conjugates 1–26 with those of 200 standard antitumor agents

Conjugate	Antitumor agents (correlation coefficient)
1	Doxorubicin (0.61)
2	Camptothecin-11 (0.61), taxotere (0.61)
3	Taxol (0.61), SN-38 (0.58)
4	Actinomycin-D (0.73), epirubicin (0.66)
5	Doxorubicin (0.68)
6	Camptothecin-11 (0.64), SN-38 (0.63)
7	SN-38 (0.54), camptothecin-11 (0.52)
8	SN-38 (0.55), actinomycin-D (0.55)
9	SN-38 (0.80)
10	SN-38 (0.74)
11	SN-38 (0.81)
12	SN-38 (0.80), mitoxantrone (0.77)
13	SN-38 (0.67)
14	Actinomycin-D (0.67), SN-38 (0.63)
15	SN-38 (0.73)
16	Melphalan (0.71), doxorubicin (0.71)
17	SN-38 (0.84), peplomycin (0.81)
18	SN-38 (0.71)
19	SN-38 (0.81), topotecan (0.80)
20	SN-38 (0.76), ICRF-193 (0.76)
21	Doxorubicin (0.81), SN-38 (0.80)
22	Neocarzinostatin (0.82), SN-38 (0.80)
23	SN-38 (0.84), doxorubicin (0.83)
24	Doxorubicin (0.76), SN-38 (0.75)
25	NK109 (0.78), SN-38 (0.76)
26	Mitomycin C (0.77), SN-38 (0.75)

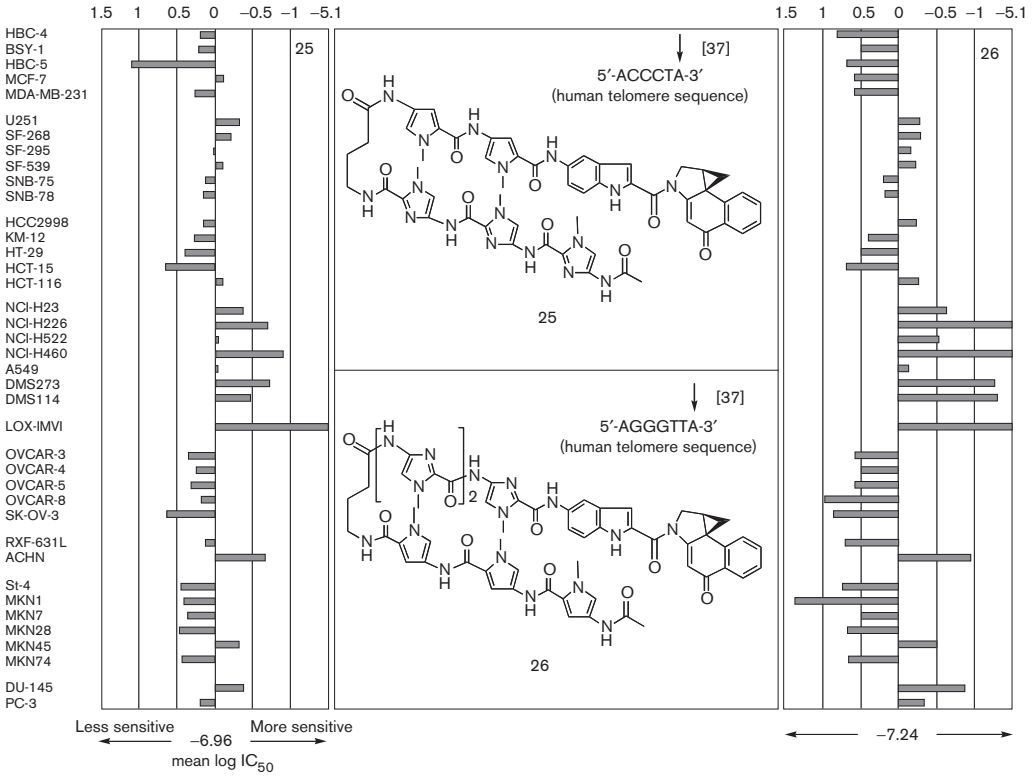
The top one or two compounds were ordered according to the correlation coefficient [24].

Although the recognition sequences of 1–26 are described as shown earlier in Figs 3–9, it would be difficult to consider that their anticancer effects are directly linked with the recognition sequences, because Py–Im diamide or triamide conjugates (5–15) can bind to target sequences through homodimer formation [27]. In contrast, hairpin-structure conjugates 17–26 alkylate their sequences strictly. Moreover, it is considered that the differences in recognition sequences and cellular permeability could affect the anticancer activities. Best *et al.* [38] showed cellular and nuclear localization of florescent Py–Im polyamides by confocal laser scanning microscopy [39]. More recently, we also evaluated the influence of the molecular size and Py–Im content of fluorescent polyamides on cellular permeability by using flow cytometry [40]. These results suggested that the efficiency of cellular permeability for Py–Im conjugates depended on a wide variety of molecular determinants, such as the molecular size of the conjugates, Py/Im contents, and the number and position of Im residues, as shown in Fig. 10.

Gene silencing by sequence-specific alkylation with pyrrole–imidazole polyamide conjugates

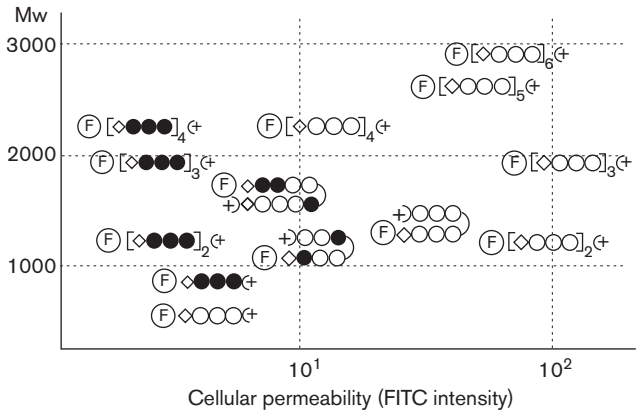
The regulation of specific gene expression by synthetic small molecules has emerged as a promising approach for the development of gene-targeting drugs. Owing to the fact that the DNA-binding affinity and sequence specificity of the Py–Im polyamides are comparable to those of a transcription factor, silencing of gene expression, such as 5S RNA, the human immunodeficiency virus, hypoxia response element and human transforming growth factor-β, was achieved by competitive binding of Py–Im hairpin polyamides to regulatory sequences [41–45]. Because gene expression is generally controlled by the binding of common transcription factors to regulatory sequences, the design of polyamides for use in this approach has certain limitations. To obtain sufficient specificity for inhibition of the expression of certain genes, Py–Im polyamides need to include unique flanking sequences of the binding sequences of transcription factors. In contrast, targeting Py–Im polyamides to unique sequences in the coding region is relatively straightforward. However, the inhibition of transcription by binding Py–Im polyamides in the coding region is difficult because the polyamides are removed from duplex DNA during polymerase II transcription. Recently, we showed that alkylating Py–Im hairpin conjugate 18, which alkylates a specific site on the template strand of the coding region of green fluorescent protein (993 bp), effectively inhibited transcription by alkylation, producing truncated mRNAs (458 nt) in an in-vitro transcription system [46]. In sharp contrast, alkylation in the nontemplate strand did not result in such truncated products. The inhibition of transcription by deactivated CPI conjugate 18 was not observed by PAGE

Fig. 9



Chemical structures of hairpin pyrrole–imidazole CBI conjugates with indole linker (25–26) and a graph for each conjugate showing the mean of 50% growth inhibition (IC_{50}) against a panel of 39 human cancer cell lines. Columns extending to the right indicate more sensitive to agents; columns extending to the left indicate less sensitive to conjugates. One unit represents one logarithmic difference.

Fig. 10



Cellular permeability for fluorescent pyrrole–imidazole polyamides by flow cytometry. HEK293 cells were incubated with each polyamide (1 μmol/l) for 24 h. (black circles: imidazole, white circles: pyrrole, β-alanine: white squares, F: fluorescein isothiocyanate).

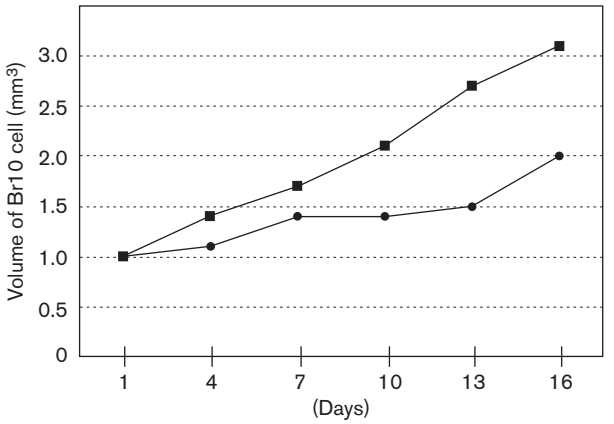
analysis, confirming that noncovalent binding does not cause inhibition of transcription. Sequence-specific gene silencing by alkylating Py–Im conjugates 20 and 21, which target the coding regions of Renilla and firefly luciferases, respectively, was investigated. Two vector plasmids were transfected into HeLa cells, and the ability to silence luciferase expression was examined *in vitro* [47]. Moreover, we showed that the indole-CBI type of alkylating polyamide can also silence the green fluorescent protein gene in living cells [48]. We expected that the alkylating Py–Im conjugates would dramatically increase its choice of target sequences as an antigene agent.

In-vivo study of pyrrole–imidazole CBI conjugate

The growth-inhibiting effects of Py–Im indole-*seco*-CBI conjugates, which target different DNA sequences, are dramatically different from those of Py–Im conjugates. These results further confirm that differences in sequence specificity might affect patterns of cytotoxicity [49]. To further study the activity of alkylating Py–Im polyamides on tumour growth *in vivo*, we used xenografts of oestrogen receptor-positive human breast cancer Br10 cells in nude mice (Fig. 11).

When the tumours were established at a size of 1 mm^3, the mice were treated with intraperitoneal injections of phosphate-buffered saline ($n=5$) or racemic conjugate 23 (50 mg/kg, n. 5) twice a week for 16 days. Owing to the

Fig. 11



Graph of the changes in tumor (Br10) volume with injections of phosphate-buffered saline (black squares) or racemic conjugate 23 (black circles).

fact that we have investigated the pharmacokinetics of simple Py–Im polyamides (no conjugates) in rats [50], it is considered that the solubility of Py–Im indole-*seco*-CBI conjugates in blood plasma would also be good. In fact, we found that the treatment with conjugate 23 significantly reduced tumour growth in Br10 xenografts.

Summary and outlook

Py–Im polyamides are attractive artificial molecules developed from detailed analysis of the DNA-recognition mechanisms of distamycin A and netropsin binding in the minor groove. They are potentially useful tools for gene regulation or functional analysis of genes because of their high sequence specificity and binding ability, which is equal to that of transcription factors. Gene expression control by Py–Im polyamides has thus been achieved by competitive binding with transcription factors in the promoter regions of genes. RNAi technology has also been developed as a useful gene regulation tool that targets specific mRNAs, large numbers of copies of which occur in the cytoplasm of cells. Therefore, complete inhibition of expression by this method over the long term is difficult, and several hurdles need to be overcome before the therapeutic use of Py–Im polyamides is possible. Our alkylating Py–Im conjugates, which target many different DNA sequences, make possible the molecular regulation of the expression of specific genes. In addition, we have successfully developed Py–Im hairpin polyamide conjugates that precisely alkylate DNA at specific matching sequences at nanomolar concentrations. The selectivity and efficiency of DNA alkylation for these conjugates is higher than that of DNA-alkylating antibiotics. The alkylating moiety CBI can be synthesized from commercially available 1,3-naphthalenediol, and the DNA-binding moiety of Py–Im polyamides can be made by solid-phase synthesis. These two functional moieties are then linked with a chemically stable indole linker. The present alkylating Py–Im polyamides can be synthesized on a large scale, which would allow for future animal studies on the development of antitumor agents targeting the expression of specific genes responsible for cancer cell growth. Examination of cytotoxicity using a nude mouse xenograft model revealed that Py–Im polyamides targeting specific sequences in individual cancer cell lines provide a promising methodology for the development of tailor-made antitumor drugs. Future studies in our laboratory will involve knowledge-based design and a combinatorial approach to identifying effective Py–Im polyamides that target the Achilles' heel of cancer cells. In addition, sequence-specific DNA-alkylating agents could become new types of specific gene silencers by alkylating specific regions in the template sequences of genes.

Interestingly, Trzupek *et al.* [51] recently showed that duocarmycin derivatives efficiently alkylate A–T base pairs, even in the nucleosome core particle-bound DNA. We also observed sequence-specific alkylation by Py–Im *seco*-CBI conjugates in nucleosome core particle DNA. These results indicate that nucleosomal DNA is fully accessible to this class of minor-groove-alkylating agents, and that the specificity and efficiency of DNA alkylation are relatively unaffected by nucleosome structure. Recently, we showed sequence-specific DNA alkylation with 10 bp recognition through heterodimeric formation. This suggests that alkylation proceeds through heterodimer formation, which would be a general way to expand the recognition sequence for DNA alkylation by Py–Im *seco*-CBI conjugates [52]. Although oligonucleotide-based agents are generating increasing excitement as potential drugs, the advantages of small molecules continues to attract interest in their development as potential gene-silencing and antitumor drugs.

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