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Sequence Specificity, Reactivity, and Antitumor Activity of DNA-Alkylating Pyrrole-Imidazole Diamides

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Summary

Three conjugates of imidazole (Im)-pyrrole (Py) diamide and a DNA-alkylating moiety derived from the antibiotic duocarmycin A were synthesized, and their sequence specificity, reactivity, and antitumor activity comparatively examined. Sequencing gel analysis indicated that ImPyDu (1) alkylates DNA at the 3′ end of AT-rich sequences at micromolar concentration. ImPyDu86 (2) reacts with DNA at AT-rich sites together with dialkylation sites at micromolar concentration. ImPyLDu86 (3) efficiently alkylates dialkylation sites at nanomolar concentration. Average values of log IC_{50} against a 39 cancer cell line panel of 1–3 were −4.59, −5.95, and −8.25, respectively. The differential growth inhibition pattern of 1–3 varied with relatively low correlation coefficients. Array-based gene expression monitoring was performed for 3 in a human lung cancer cell line. Substantial downregulation of expression was seen for genes involved in DNA damage response, transcription, and signal transduction.

Introduction

DNA-alkylating agents have long been of interest for their biological properties. They constitute a major class of such antitumor drugs as nitrosoureas, mitomycin C, cisplatin, and nitrogen mustards, and they are routinely used in hospitals. Many alkylating antitumor agents are, in fact, practically nonselective and cannot distinguish between cancer cells and normal cells; as a result, they exhibit severe toxicity to normal tissues. Recently, it has become evident that DNA sequence specificity is an important component contributing to the cytotoxic potency of several antitumor agents [1, 2]. Therefore, the question arises whether one can tailor the binding preference of DNA binding agents to particular sequences and thereby create a tailor-made antitumor agent. The study of DNA minor groove binders has emerged as a methodology for the development of novel antitumor agents with sequence recognition ability. On the basis of earlier work on the concept of minor groove sequence information readout [3–8], Dervan and colleagues have developed minor-groove binding hairpin polyamides containing *N*-methyl pyrrole (Py)-*N*-methyl imidazole (Im) that uniquely recognize each of the four Watson-Crick base pairs [9–15]. A pairing of Im opposite Py targets a G-C base pair, while Py/Im targets a C-G base pair. Py/Py degenerately targets T-A and A-T base pairs [16–18].

Duocarmycin A (Duo) [19–21] is an exceedingly potent antitumor antibiotic isolated from *Streptomyces sp*. that selectively alkylates N3 of adenine (A) at the 3′ end of consecutive A·T base pairs in DNA [22]. Several years ago, we found that the addition of distamycin A (Dist) causes efficient alkylation at the G residues in GC-rich sequences [23] through a cooperative heterodimer formation between Duo and Dist in the minor groove. This suggests that Dist recognizes the complementary strand according to a similar binary code of Py-Im polyamides. We therefore believed that Py-Im polyamides could be used as recognition components to deliver alkylating agents to a target sequence. Thus, we prepared the hybrid molecules constructed from segment A of Duo and Py-Im polyamides [24, 25]. It was shown that these molecules specifically alkylate at predetermined sequences within a 450 bp DNA fragment [25]. Lown and colleagues have found that insertion of a *trans*-vinyl linker (L) between the Py and CPI groups greatly enhanced the alkylating activity, as well as the cytotoxicity [26]. Based on this observation, we synthesized Py-Im diamide CPI conjugates with a vinyl linker, ImPyLDu86 (**3**), and demonstrated that **3** efficiently causes double alkylation of DNA at the 5′-PyG(A/T)CPu-3′/5′-PyG(A/T)CPu-3′ sequence through a highly cooperative homodimer formation [27]. We found that the efficiency of alkylation by **3**, which is the amount of DNA alkylation divided by the amount of the agent, was 69%, thus confirming the unusually high efficiency of dialkylation.

In developing an efficient sequence-specific alkylating agent, we found that the choice of alkylating moiety and linker region are very important. We describe here a comparative study of DNA sequence-specific alkylation and antitumor activity of three different alkylating ImPy diamide conjugates **1–3** utilizing high-resolution denaturing gel electrophoresis and a panel of 39 human cancer cell lines. To gain insight into the origin of the intense antitumor activity of ImPyLDu86 (**3**) expression level, a gene chip analysis was also performed.

Results and Discussion

Synthesis and Solvolytic Stability of ImPy Diamide Conjugates 1–3

We synthesized ImPy diamide conjugates **1** and **2** as shown in Figure 1. Carboxylic acid **4** was activated with

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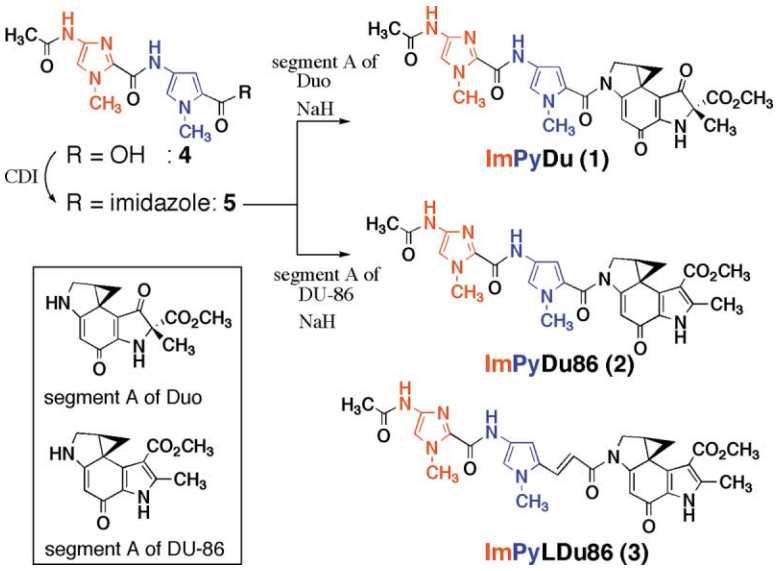

Figure 1. Structures and Synthesis of Conjugates **1**, **2**, and **3**

CDI to **5**, which was coupled with segment A of Duo or DU-86 to provide **1** and **2**, respectively. Segment A of Duo and DU-86 was prepared according to the reported procedures [28]. Compound **3** was prepared by previously reported procedures [27]. The conjugates **1–3** were purified by reverse phase HPLC and subjected to DNA alkylation and cytotoxicity experiments. The solvolytic stability of **1–3** was examined in 5 mM sodium phosphate buffer (pH 7.0) containing 20% DMF at 37°C by monitoring the disappearance of **1–3** by HPLC. It was observed that the half-lives of **1–3** are 3, 53, and 53 hr, respectively, indicating that the extension of conjugation greatly stabilizes the solvolytic stability. Enhanced solvolytic stability of **2** and **3** relative to **1** is consistent with the previous observation of Duo compared with duocarmycin SA [29].

Evaluation of DNA Alkylation by 1–3 Using 450 bp DNA Fragments

Sequence-selective alkylation by compounds **1–3** was investigated on 5′ Texas red-labeled 450 bp DNA fragments using an automated DNA sequencer, as previously described [24, 25, 27]. Alkylation was carried out at 23°C for 24 hr and quenched by the addition of calf thymus DNA. Samples were heated at 94°C under neutral conditions for 20 min. The sites of alkylation were visualized by thermal cleavage of the DNA strand at the alkylated sites. Under these heating conditions, all purine N3-alkylated sites in the DNA produced cleavage bands almost quantitatively on the gel. Subsequent hot piperidine treatment (0.1 M, 90°C, 20 min) did not further enhance the cleavage bands, indicating that the neutral heating conditions used (94°C, 20 min) were sufficient to cleave all the DNA-alkylated sites. Sequencing analysis of the alkylated DNA fragments after heat treatment is shown in Figure 2.

Alkylation of 10 nM of the DNA fragment by **1** or **2** was observed at 100 μM concentration of the agent. Several alkylations by **1** occurred predominantly at the 3′ end A of AT-rich sequences, 5′-AAAA-3′ (site 1), 5′-TAAA-3′ (site 2), 5′-ACAA-3′ (site 3), 5′-ATAA-3′ (site 7) (lanes 1–4). These results clearly indicate that **1** alkylates DNA in a simple monomeric binding mode and that the ImPy diamide moiety of **1** does not effectively recognize G-C base pairs except for the alkylation at site 3, which could be explained by recognition of Im in the monomeric binding mode reported by Dervan et al. [30]. Interestingly, alkylation by **2** occurred at specific sequences in close proximity, such as 5′-CGACA-3′ (site 4)/5′-TGTCG-3′ (site 10), 5′-CGACG-3′ (site 5)/5′-CGTCG-3′ (site 9), 5′-TGACG-3′ (site 6)/5′-CGTCA-3′ (site 8), together with AT-rich sites as observed in the case of **1** (lanes 5–8). These results clearly indicate that substitution of the alkylating moiety with segment A of the DU-86 unit increased the planarity of the molecule, which allows double alkylation of DNA at 5′-PyG(A/T)CPu-3′/5′-PyG(A/T)CPu-3′ through cooperative homodimer formation. As previously reported, **3**, which possesses a vinyl linker relative to **2**, alkylated the 5′-PyG(A/T)CPu-3′ (sites 4–6 and 8–10) at a concentration of 100−10 nM of **3** (lanes 9–12). The significantly improved reactivity of **3** could be attributed to highly cooperative homodimer formation in the DNA minor groove. These results confirmed that incorporation of a vinyl linker paired with Im substantially improves the reactivity of ImPy diamide Du86 conjugates. Therefore, these results also suggest that **3** effectively alkylates DNA according to the Py-Im pairing rule, and exhibits a new mode of recognition in which the Im-vinyl linker (L) pair targets G-C base pairs.

Evaluation of 50% Growth Inhibition by 1–3 against a Panel of 39 Cancer Cell Lines

The Cancer Chemotherapy Center in Japan has established a new human cancer cell line panel, which consists of 39 human cancer cell lines [31, 32], coupled with a drug sensitivity database that is similar to the National Cancer Institute cancer cell line panel [33–35]. New compounds can be compared with standard drugs for the 50% cell growth inhibition (IC_{50}) mean graphs using COMPARE analysis and examined to see if new com-

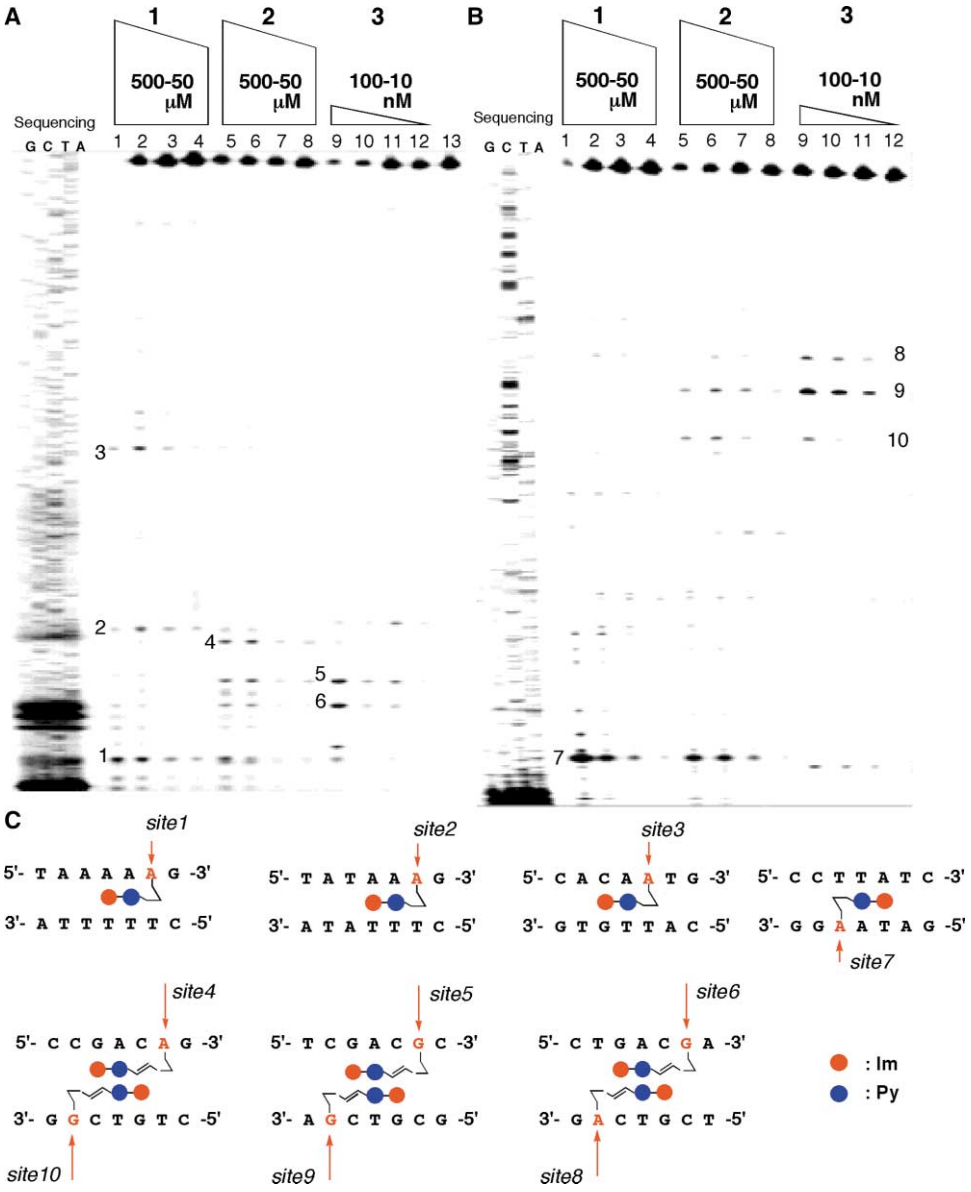

Figure 2. Sequence Specific DNA-Alkylation by Conjugates **1**, **2**, and **3**

Thermally induced strand cleavage of 5′ Texas red-labeled 450 bp DNA fragments by conjugates **1–3**. Results using 5′ end-labeled top strand (pUC 18 F780−1229) (A) and 5′ end-labeled bottom strand (pUC 18 R1459−1908) (B) DNA fragments are shown. These two DNA fragments are complementary. Lanes 1–4: 500, 200, 100, and 50 μM of **1**; lanes 5–8: 500, 200, 100, and 50 μM of **2**; lanes 9–12: 100, 50, 25, and 10 nM of **3**; lane 13: DNA control. Sequences containing alkylation sites are represented (C). Arrows indicate the site of alkylation by **1–3**. Alkylated bases are shown in red.

pounds having unique or similar mechanisms are clustered together. In general, the panel patterns of drugs possessing a common mechanism resembled one another ($r > 0.75$).

Using this human cancer cell line panel, we investigated the IC_{50} profile of the ImPy diamide CPI conjugates **1–3**, as shown in Figure 3. The mean log IC_{50} of **1** was −4.59 (25.7 μM), which is comparable with the presently used anticancer drug 5-fluorouracil. In contrast, the mean log IC_{50} of **2** was increased to −5.95 (1.12 μM) because of its high stability in the cell. Interestingly, the mean log IC_{50} of **3** was significantly increased to −8.25 (5.62 nM), which can be attributed to dramatic improvement of DNA alkylation activity by the insertion of a vinyl linker between the minor groove binder and the alkylating moiety. The COMPARE analysis of the mean graphs showed that the ImPy diamide conjugates **1–3** did not correlate well with each other ($r = 0.55–0.68$) despite having a common DNA-alkylating mechanism. It is generally accepted in a COMPARE analysis that higher correlation coefficients ($r > 0.75$) are observed for anticancer agents possessing the same reaction mechanism. For example, the correlation coefficient between doxorubicin and daunorubicin (DNA intercalater)

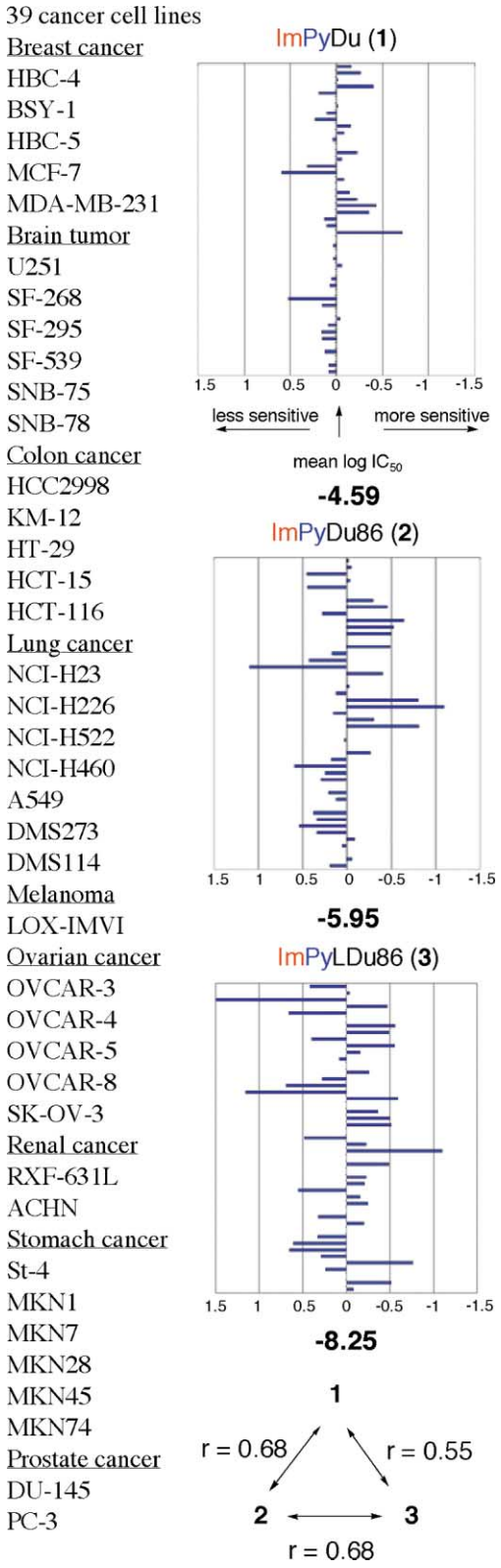

Figure 3. The Mean Graphs of 50% Growth Inhibition against a Panel of 39 Human Cancer Cell Lines

The log IC_{50} for each cell line is indicated. Columns extending to the right, more sensitive to agents; columns extending to the left, less sensitive to agents. One unit represents one logarithm difference.

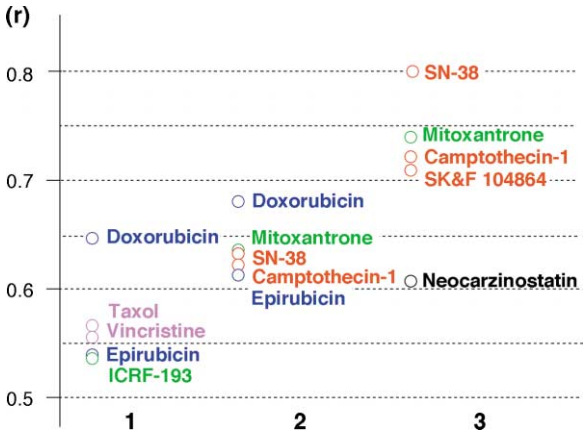

Figure 4. The Results of Compare Analysis of **1**–**3** with Those of 200 Standard Antitumor Agents

The top five compounds were ordered according to the correlation coefficient. Doxorubicin, Epirubicin (DNA intercalater) were drawn in blue. SN-38, Camptothecin-1, SK&F 104864 (Topo I inhibitor) were drawn in red. Mitoxantrone, ICRF-193 (Topo II inhibitor) were drawn in green. Taxol, Vincristine (tubulin inhibitor) were drawn in purple. Neocarzinostatin (DNA damage) was drawn in black.

is 0.93 [36]. The value for ecteinascidin Et 743 and phthalascidin Pt 650 (guanine N2 alkylation) is 0.90 [37]. Therefore, the correlation coefficients between **1**, **2**, and **3** (purine N3 alkylation), which are in the range 0.55–0.68, are remarkably low. These results suggest that the difference in sequence specificity of **1**–**3** may reflect the pattern's difference in the mean graphs and relatively low correlation coefficient with one another. Sequence specificity of alkylating Py-Im polyamide can be changed in a predictable manner; therefore, a sequence-specific alkylating agent targeting a predetermined specific sequence in cancer cells will be a promising approach for the development of tailor-made antitumor drugs.

By comparing with the result of the screening panel of existing anticancer drugs, it became clear that an interesting tendency existed as shown in Figure 4. The chemical compound that showed the highest correlation coefficient of the screening panel of **1** and **2** was the DNA intercalating agent, doxorubicin. For both **3** and **2**, mitoxantrone, which is a Topoisomerase II inhibitor, came second. The first resembled compound for **3** was found to be Topoisomerase I inhibitor SN38, which was the third for **2**. The third resembled compound for **3** was camptothecan, which was the fourth for **2**. These results indicate that resemblances of the screening panel roughly corresponds to the DNA binding modes with which **1** and **3** alkylate DNA as the monomer and dimer, respectively, and **2** possesses both properties.

Analysis of Gene Expression Profiles by ImPyLDu86 (3) Using a Gene Chip

Since conjugate **3** shows intense cytotoxicity against human cancer cell lines, array-based gene expressions were performed using HLC-2. It was found that incubation of 1 nM of **3** induced downregulation of 123 genes (>2-fold) and 85 genes (>4-fold) among 2069 genes, as shown in Figure 5. It is important to note that only 1 nM

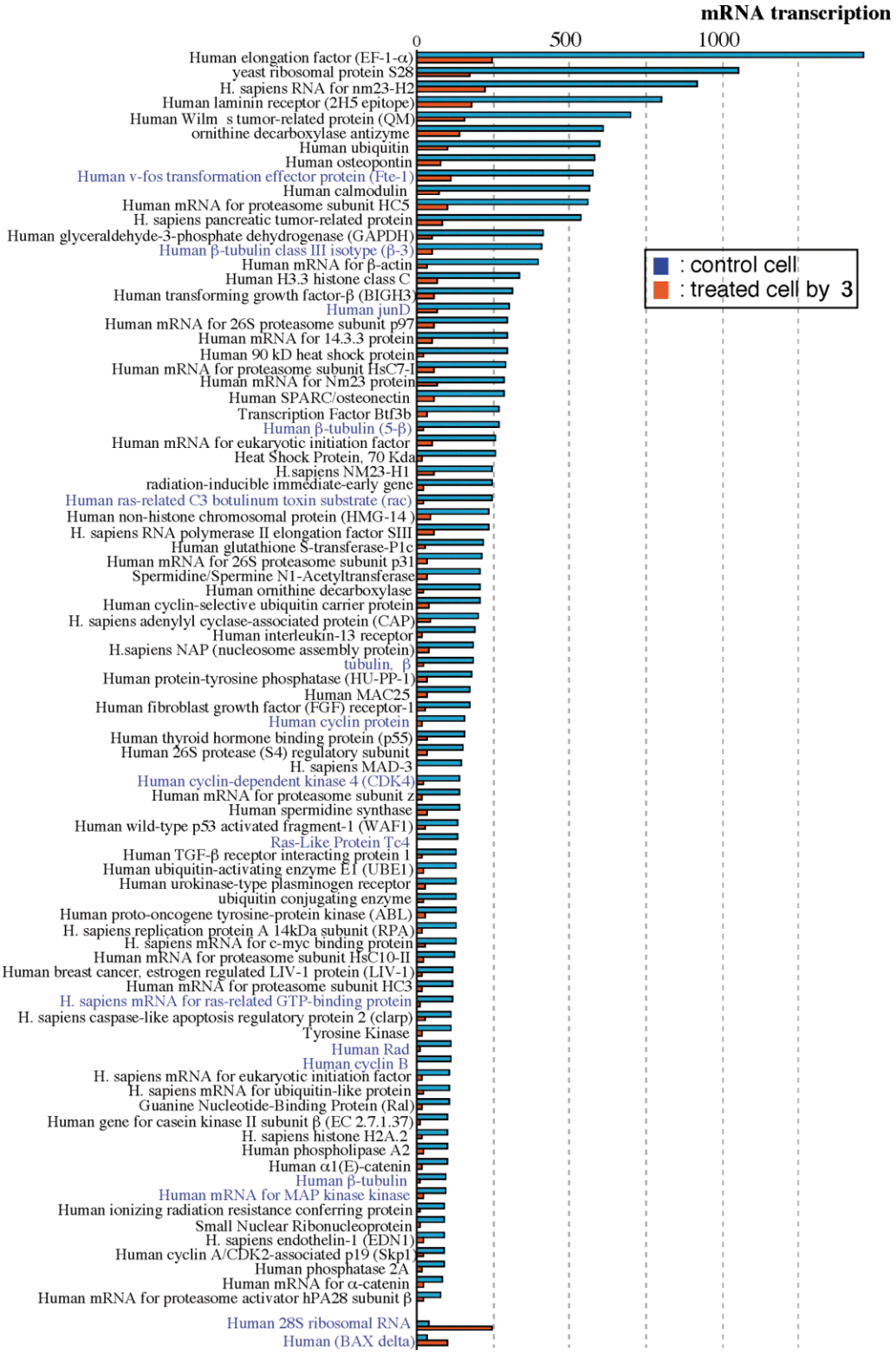

Figure 5. 87 Columns Comparing Sets of Downregulated (85 genes) and Upregulated (2 genes) Genes Using ImPyLDu86 (3) in the HLC-2 Lung Cancer Cell

The transcription amounts of m-RNA in control cells are drawn in the blue column, and those in drug-treated cells are drawn in the red column.

Table 1. Gene Expression Changes Caused by ImPyLDu86 (3)

Accession No.	Treated Cell	Control Cell	Fold Change	Gene Definition
Downregulated Genes (85/2069 Genes)				
M29870	21	245	−11.3	Ras-related C3 botulinum toxin substrate (Rac)
	8	134	−17.4	Ras-like protein Tc4
L24564	14	110	−7.9	Rad
D78132	11	117	−10.3	Ras-related GTP-binding protein
D87116	22	95	−4.3	MAP kinase kinase
X56681	69	303	−4.4	junD
M84711	112	579	−5.1	v-fos transformation effector protein
M25753	7	109	−14.7	Cyclin B
	14	157	−11.0	Cyclin protein
U37022	20	142	−6.9	Cyclin-dependent kinase 4 (CDK4)
U33760	22	89	−4.0	Cyclin A/CDK2-associated p19
	23	184	−8.1	β-tubulin
J00314	13	97	−7.5	β-tubulin
U47634	53	406	−7.7	β-tubulin class III isotype (β-3)
X00734	20	267	−13.5	β-tubulin 5-β
Upregulated Genes (2/2069 Genes)				
M27830	245	37	6.6	28S ribosomal RNA
U19599	103	31	3.3	BAX delta

of 3 is needed to exert such significant effects on the gene expression after 1 hr.

The results of regulated genes summarized in Table 1 show that 3 affects many sets of gene expression, which is related to DNA-alkylating damage response, transcription, and signal transduction. Especially dramatic changes were observed in subsets of genes involved in Ras, cyclin B, and tubulin β. The level of gene expressions were observed to be less than 1/10 relative to that of the control cells. A concentration of only 1 nM of 3 for 1 hr seriously damaged DNA in the cancer cell. The inhibition of Ras gene expression results in the inhibition of MAPKK, jun, and fos in the downstream of gene expression. The high inhibition of these gene transcriptions might cause inhibition of cell growth and cell division by DNA alkylation. In contrast, only two upregulated genes (> 2-fold), human BAX delta and 28S ribosomal RNA, were observed in the HLC-2 cell. A possible explanation for the characteristic upregulation is that 3 may specifically activate transcription for apoptosis in this cancer cell.

Significance

To date, various types of minor groove alkylating agents have been synthesized, but the relationship between sequence specificity and antitumor activity is not well understood. In this study, three different conjugates of ImPy diamide and an alkylating moiety derived from the antibiotic Duo were comparatively examined. Sequence specificity and reactivity of ImPy diamide conjugates 1–3 dramatically depend on the structure of the alkylating agent and the linker region between the alkylating moiety and the recognition moiety. Antitumor activity of 1–3 was examined using a 39 human cancer cell line panel coupled with a drug sensitivity database. Average logs IC_{50} against the 39 cancer cell line panel of 1–3 were −4.59, −5.95, and −8.25, respectively. The results clearly indicate that the antitumor activities of 1–3 can be explained by the combination of alkylating activities together with their solvolytic stabilities. The differential growth inhibition patterns of 1–3 varied, with relatively low correlation coefficients among the responding cell lines, implying that sequence specificity might affect antitumor activity toward certain cancer cell lines. Array-based gene expression monitoring was performed for 3 in the human lung cancer cell line HLC-2. Characteristic changes were observed in subsets of genes involved in DNA damage response, transcription, and signal transduction. To the best of our knowledge, this is the first gene array analysis of alkylating Py-Im polyamide, and sets a minimum baseline for evaluation. The present results suggest the intriguing possibility that the effective DNA-alkylating agent recognized for longer base pair sequences may provide a promising approach for developing new types of biological agents to control gene expression [38, 39]. This paves the way for a "tailor-made antitumor agent" by sequence-specific alkylating Py-Im polyamides.

Experimental Procedures

Chemicals

Reagents and solvents were purchased from standard suppliers and used without further purification. Abbreviations of some reagents: CDI, 1,1′-carbonyldiimidazole; DMF, *N,N*-dimethylformamide. Reactions were monitored by thin-layer chromatography (TLC) using 0.25 mm silica gel 60 plates impregnated with 254 nm fluorescent indicator (from Merck). Plates were visualized by UV illumination. NMR spectra were recorded with a JEOL JNM-A 500 nuclear magnetic resonance spectrometer, and tetramethylsilane was used as the internal standard. Proton NMR spectra were recorded in parts per million (ppm) downfield relative to tetramethylsilane. The following abbreviations apply to spin multiplicity: s, singlet; d, doublet; m, multiplet; and br, broad. Electrospray ionization mass spectra (ESMS) were produced on a PE SCIEX API 165 mass spectrometer. Polyacrylamide gel electrophoresis was performed on a HITACHI 5500-S DNA Sequencer. Ex Taq DNA polymerase and Suprec-02 purification cartridges were purchased from Takara Co., the Thermo Sequenase core sequencing kit and loading dye (di-

methylformamide with fuchsin red) from Amersham Co. Ltd, 5′ Texas red-modified DNA oligomer (18-mer) from Kurabo Co. Ltd and 50% Long Ranger gel solution from FMC Bioproducts. The following drugs were synthesized by the reported procedures [24, 25]. **ImPyDu** (**1**): ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.68 (s, 1H; NH), 7.68 (brs, 1H; NH), 7.33 (s, 1H; CH), 7.31 (d, J = 1.5 Hz, 1H; CH), 6.51 (d, J = 1.5 Hz, 1H; CH), 6.43 (s, 1H; CH), 6.10 (s, 1H; NH), 4.19 (dd, J = 5 Hz, 11 Hz, 1H; NCH*H*), 4.01 (d, J = 11 Hz, 1H; NC*H*H), 3.98 (s, 3H; NCH_3), 3.78 (s, 3H; NCH_3), 3.67 (s, 3H; OCH_3), 2.86 (m, 1H; CH), 2.22 (m, 1H; CH*H*), 2.10 (s, 3H; $COCH_3$), 1.60 (s, 3H; CH_3), 1.24 (m, 1H; C*H*H); ESMS *m/z* calcd for $C_{27}H_{28}N_7O_7$ [M^+ + H] 562.2, found 562.1. **ImPyDu86** (**2**): ^{1}H NMR (500 MHz, [D_6]DMSO) δ 12.36 (s, 1H; NH), 10.18 (s, 1H; NH), 9.98 (s, 1H; NH), 7.46 (d, J = 1.5 Hz, 1H; CH), 7.42 (s, 1H; CH), 6.86 (d, J = 1.5 Hz, 1H; CH), 6.16 (s, 1H; CH), 4.24 (dd, J = 5 Hz, 11 Hz, 1H; NCH*H*), 4.08 (d, J = 11 Hz, 1H; NC*H*H), 3.94 (s, 3H; NCH_3), 3.73 (s, 6H; NCH_3 and OCH_3), 3.41 (m, 1H; CH), 2.47 (s, 3H; CH_3), 2.17 (m, 1H; CH*H*), 2.02 (s, 3H; $COCH_3$), 1.40 (m, 1H; C*H*H); ESMS *m/z* calcd for $C_{27}H_{28}N_7O_6$ [M^+ + H] 546.2, found 546.1.

Preparation of 5′ Texas Red-Modified 450 bp DNA Fragments

The 5′ Texas red-modified 450 bp DNA fragments pUC18 F780*-1229 and pUC18 R1459*-1908 (these two DNA fragments are complementary) were prepared by PCR using 5′ Texas red-modified 20-mer primers: 5′-AGAATCAGGGGATAACGCAG-3′ (pUC18 forward, 780–799) and 5′-TTACCAGTGGCTGCTGCCAG-3′ (pUC18 reverse, 1459–1478). Fragments were purified by filtration using Suprec-02, and their concentrations were determined by UV absorption. The asterisk indicates Texas red modification and the nucleotide numbering starts with the replication site.

High-Resolution Gel Electrophoresis

The 5′ Texas red-labeled DNA fragments (10 nM) were alkylated by various concentrations of **1–3** in 10 μl of 5 mM sodium phosphate buffer (pH 7.0) containing 10% DMF at 23°C. The reaction was quenched by the addition of calf thymus DNA (1 mM, 1 μl) and heating for 5 min at 90°C. The DNA was recovered by vacuum centrifugation. The pellet was dissolved in 8 μl loading dye (formamide with fuchsin red), heated at 94°C for 20 min, and then immediately cooled to 0°C. A 2 μl aliquot was subjected to electrophoresis on a 6% denaturing polyacrylamide gel using a Hitachi 5500-S DNA Sequencer.

Analysis of Growth Inhibition against 39 Human Cancer Cell Lines

The human cancer cells were plated at an appropriate density in 96-well plates in RPMI 1640 with 5% fetal bovine serum and allowed to attach overnight. The cells were exposed to drugs for 48 hr. Then, the cell growth was determined according to the sulforhodamine B assay. Absorbance for the control well (C), the treated well (T) and the treated well at time 0 (T_0) were measured at 525 nm. The 50% growth inhibition (IC_{50}) was calculated as $100 \times [(T - T_0)/(C - T_0)] = 50$. The mean graphs, which show the differential growth inhibition of the drugs in the cell line panel, were drawn based on a calculation using a set of IC_{50}. Pearson correlation coefficients (*r*) were calculated using the following formula: $r = [\Sigma(x_i - x_m)(y_i - y_m)]/[\Sigma(x_i - x_m)^2\Sigma(y_i - y_m)^2]^{1/2}$, where x_i and y_i are log IC_{50} of drug A and drug B, respectively, against each cell line, and x_m and y_m are the mean values of x_i and y_i, respectively.

Microarray Analysis

HLC-2 cells were cultured in 150 ml bottles with 30 ml fresh medium at 1.0×10^6 cells per bottle. After 24 hr preincubation, the cells were treated with 1 nM ImPyLDu86 (**3**) in 0.01% DMSO or with 0.01% DMSO alone as control for 1 hr. HLC-2 cells were washed with ice-cold RNase-free phosphate-buffered saline (PBS). mRNA was extracted directly with oligo-dT cellulose using the Quick Prep Micro mRNA Purification Kit (Amersham Pharmacia Biotech, Buckinghamshire, UK) in accordance with the manufacturer's instructions. Experiments with GeneChips (Affymetrix, Santa Clara, CA) were performed according to the manufacturer's instructions [40, 41]. Double-stranded cDNA was synthesized by reverse transcription performed with the Superscript Choice System (GIBCO Life Technologies, Gaitherburg, MD) from mRNA extracted from HLC-2 cells. The resulting cDNA was purified by phenol/chloroform extraction with Phase Lock Gel (5′ to 3′, Inc. Boulder, CO), and concentrated by ethanol precipitation. Synthetic double-stranded cDNA transcription into biotin-labeled cRNA was done in vitro with a MEGAscript T7 kit (Ambion, Austin, Texas). Biotin-labeled cRNA was then isolated with an RNeasy Mini Kit (Qiagen, Tokyo, Japan) and precipitated with ethanol. The cRNA was fragmented to 50–200 nucleotide pieces as described by Wodicka et al. [42]. After fragmentation, 5 μg of cRNA was injected into a Human Cancer G110 Array probe array cartridge (Affymetrix), which contains probe sets for 2069 genes including ca. 1700 human cancer related genes for hybridization. The G110 array contains oligonucleotides representing transcripts of metabolic enzymes, growth factors and receptors, kinases and phosphatases, nuclear receptors, transcription factors, DNA damage repair genes, apoptosis genes, stress response genes, membrane proteins, and cell cycle regulators. Probe arrays were treated with biotinylated anti-streptavidin goat antibody (Vector Laboratories, Burlingame, CA) and stained with streptavidin phycoerythrin (Molecular Probes, Eugene, OR). Probe arrays were scanned twice with a GeneChip scanner at a resolution of 3 μm. The intensity for each feature of the array was captured with Affymetrix GeneChip Expression Analysis Software according to standard Affymetrix procedures, and the gene expression data were analyzed with Microsoft Excel. Expression values of transcription in different groups were normalized by adjusting GAPDH expression as 5000 to the same value. The final values for each transcription as increments were the mean of duplicate values for treated HLC-2 cells minus the average values for nontreated HLC-2 cells. All quantitative data were processed using the Affymetrix GeneChip software. 4-fold downregulated expression and 2-fold upregulated expression were shown in Figure 5.

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