A rhodium(III) complex for high-affinity DNA base-pair mismatch recognition

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A rhodium(III) complex, rac-[Rh(bpy)2phzi]3⁺ (bpy, 2,2'-bipyridine; phzi, benzo[a]phenazine-5,6-quinone diimine) has been designed as a stericly demanding intercalator targeted to destabilized mismatched sites in double-helical DNA. The complex is readily synthesized by condensation of the phenazine quinone with the corresponding diammine complex. Upon photo-activation, the complex promotes direct strand scission at single-base mismatch sites within the DNA duplex. As with the parent mismatch-specific reagent, [Rh(bpy)2(chrysi)]3⁺ [chrysen-5,6-quinone diimine (chrysi)], mismatch selectivity depends on the helix destabilization associated with mispairing. Unlike the parent chrysi complex, the phzi analogue binds and cleaves with high affinity and efficiency. The specific binding constants for CA, CC, and CT mismatches within a 31-mer oligonucleotide duplex are 0.3, 1, and 6 × 10⁸ M⁻¹, respectively; site-specific photocleavage is evident at nanomolar concentrations. Moreover, the specificity, defined as the ratio in binding affinities for mispaired vs. well paired sites, is maintained. The increase in affinity is attributed to greater stability in the mismatched site associated with stacking by the heterocyclic aromatic ligand. The high-affinity complex is also applied in the differential cleavage of DNA obtained from cell lines deficient in mismatch repair vs. those proficient in mismatch repair. Agreement is found between photocleavage by the mismatch-specific probes and deficiency in mismatch repair. This mismatch-specific targeting, therefore, offers a potential strategy for new chemotherapeutic design.

The targeting of single-base mismatches in DNA represents a challenging problem in molecular recognition. The mismatched site may be of variable sequence and within a variable sequence context. What distinguishes such a site, instead, is the local destabilization in opposing bases because of the absence of Watson–Crick hydrogen bonding (1). Although challenging, however, the development of small molecules targeted to mismatches offers many applications. Site-specific mismatch probes could be used in discovery efforts to identify single-nucleotide polymorphisms. Moreover, such molecules could provide the basis for the development of novel chemotherapeutics. Many cancers are associated with a deficiency in mismatch repair (2, 3). Hence, by directing small molecules to the accumulated mismatches, a cancer-specific targeting strategy could be envisioned.

In our laboratory, we have exploited the local helix destabilization associated with mispairing in the design of a metal complex targeted to mismatches (4, 5). Shown in Fig. 1 is a rhodium complex containing the benzo[a]phenazine-5,6-quinone diimine (phzi) ligand, as well as chrysene-5,6-quinone diimine (chrysi) and phenanthrene quinone diimine (phi) ligands (4). Octahedral rhodium(III) complexes containing the phi ligand bind avidly to double-helical DNA by intercalation (6); on photoactivation, direct DNA strand cleavage is also promoted at the bound site (7). By tuning the ancillary ligands, complexes can be prepared that are either site-specific or sequence-neutral. [Rh(bpy)2(phi)]3⁺ (bpy, 2,2'-bipyridine), for example, binds to B-DNA with little site selectivity (7), whereas [Rh(S,S-dimethyltrien)(phi)]3⁺ specifically targets the sequence 5'-TGCA-3' (8). The high-resolution crystal structure of [Rh(S,S-dimethyltrien)(phi)]3⁺ bound to a DNA octamer shows site-specific intercalation by the complex from the major groove side of the helix, with site-disentanglement determined through an ensemble of hydrogen bonding contacts and methyl–methyl interactions (9). The intercalated phi ligand itself resembles another base pair, stacked at a distance of 3.4 Å between neighboring base pairs, with an expanse across the ligand of dimensions matching that of the base pairs above and below.

The chrysi complex represents our first-generation complex targeted to mismatches (4). The site-specificity of the complex is derived from the fact that it is a more bulky intercalator with an expanse exceeding that of the well matched base pair (Fig. 1). Owing to shape selection, the complex is unable to bind to well matched B-form DNA. However, at sites where a base pair is destabilized, intercalation of the chrysi complex can occur. [Rh(bpy)chrysi]3⁺ has been shown to be both a general and a remarkably specific mismatch recognition agent (10, 11). Specific DNA cleavage is observed at >80% of mismatch sites in all of the possible single base-pair sequence contexts around the mispaired bases. Moreover, the complex was found to recognize and photocleave at a single base mismatch in a 2,725-bp linearized plasmid heteroduplex.

Here, we describe the synthesis and application of a second generation mismatch recognition agent, [Rh(bpy)2phzi]3⁺. This complex shares with the chrysi complex a sterically demanding intercalating ligand. However, the phi ligand, as an aromatic heterocycle, offers the possibility of greater stabilization through stacking within the mismatched site. Indeed, [Rh(bpy)2phzi]3⁺ binds mismatched sites with a binding affinity increased by two orders of magnitude, without a loss in site-selectivity. This high affinity allows the application of the complex in the differential targeting of DNA in a mismatch-repair-deficient cell line.

Materials and Methods

Materials. Commercially obtained chemicals were used as received. Bipyridine, triflic acid, 2,3-dichloro-1,4-naphthoquinone, and phenylenediamine were purchased from Aldrich. RhCl₃-2H₂O was obtained from Pressure Chemical (Pittsburgh). Benzo[a]phenazinequinone was synthesized as described (12).

Instrumentation. Electronic spectra were recorded on a Beckman DU 7400 UV-visible spectrophotometer (Beckman Coulter). Mass spectra [electrospray ionization (ESI)] were measured on an LCQ mass spectrometer. High-performance liquid chromatography was performed on a Waters 996 system using a Vydac C18 column (4.0 ml/min liquid phase, linear gradient over 50 min from 100% 50 mM NH₄OAc, pH 4 to 100% acetonitrile).

Abbreviations: phi, benzo[a]phenazine-5,6-quinone diimine; chrysi, chrysene-5,6-quinone diimine; phi, phenanthrene quinone diimine; bpy, 2,2'-bipyridine.

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Seppac-C18 column, eluted with a mixture of acetonitrile, water, and TFA (1:1:0.001), and lyophilized to dryness. The yield was 23 mg (30% yield). Microanalysis found (calculated): C 54.8 (55.4), H 3.6 (3.3), N 15.3 (14.8). (1)H NMR (d8-DMSO, 300 MHz): δ 14.88 (s), 14.70 (s), 9.03 (m, 4H), 8.90 (d, 2H, 7.2 Hz), 8.72 (d, 1H, 6.1 Hz), 8.60 (t, 2H, 8.5 Hz), 8.54 (d, 1H, 6.1 Hz), 8.47 (t, 2H, 8.5 Hz), 8.32 (d, 1H, 8.5 Hz), 8.20 (d, 1H, 8.5 Hz), 8.11 (t, 1H, 7.3 Hz), 8.03 (m, 3H), 7.94 (t, 1H, 6.1Hz), 7.84 (t, 1H, 7.3Hz), 7.75 (m, 3H), 7.69 (d, 1H, 6.1 Hz) ppm. UV/vis (H2O, pH 5): 245 nm (89,800 M⁻¹ cm⁻¹), 304 nm (65,800 M⁻¹ cm⁻¹), 314 nm (67,300 M⁻¹ cm⁻¹), 343 nm (39,300 M⁻¹ cm⁻¹). ESI-MS (cation) 671(M+H+)

DNA Preparation and Photocleavage Experiments. The oligonucleotides, 5'-GAT GTC GCT CCC ACC ATG AGC GAT TAC C-3' and 5'-GAG TTG GTA ATC CGT CAC CAT CGT GCG ACC GAC ATC ATG CG-3', where C denotes the position of the mismatch, were synthesized on an ABI 392 DNA/RNA synthesizer (Applied Biosystems) by using standard phosphoramidite solid-phase synthesis; they were initially purified on PolyPak II cartridges and further purified by HPLC (98% 100 mM NH₄OAc/2% acetonitrile to 70% 100 mM NH₄OAc/30% acetonitrile over 30 min). The single strands were then 5'-labeled with [γ-32P]ATP and T4 polynucleotide kinase. The labeled strands were further purified by gel electrophoresis (20% denaturing polyacrylamide gel), eluted from the gel by soaking in triethylammonium acetate (100 mM, pH 7.0), ethanol precipitated, and annealed in the presence of unlabeled DNA. To the labeled 31-mer duplex (2 μM in 50 mM NaCl/10 mM Tris-HCl, pH 8.5) was added either [Rh(bpy)2(phzi)]Cl₃ or [Rh(bpy)2chrysi]Cl₃, and the sample was irradiated. After irradiation, all samples were lyophilized, denaturing formamide loading dye was added, and the samples were electrophoresed on a 20% polyacrylamide denaturing gel. The photocleavage results were quantitated by phosphorimagery (PhosphorImager, Molecular Dynamics).

Mismatch-Repair-Proficient and -Deficient Cell Lines and Photocleavage of Genomic DNA. Cell lines were obtained from the National Cancer Institute cell repository (National Cancer Institute-Frederick Cancer Research and Development Center, Frederick, MD). DNA was isolated according to standard methods (15). Isolated DNA was digested with EcoRI, dephosphorylated with shrimp alkaline phosphatase (SAP), and radiolabeled with [γ-32P]ATP, as follows: 100 μl of 100 μM solution of DNA from each cell line contained 10 μl of 10× EcoRI buffer, to which 5 μl of EcoRI (NEB, Beverly, MA) was added. The reactions were allowed to incubate at 37°C for ~8 h. The solutions then were put through Bio-Rad microfuge exchange spin columns and subsequently treated with 10 μl of 10× SAP buffer and 7 μl of SAP. The solutions were allowed to incubate at 37°C for at least 4 h before deactivation by heating at 65°C for 15 min. The solutions then were put through Bio-Rad exchange columns once again and subsequently treated with 10 μl of 10× T4 polynucleotide kinase (PNK buffer: 70 mM Tris-HCl, pH 7.6/10 mM MgCl₂, 5 mM DTT), 3 μl of [γ-32P]ATP (ICN) and 5 μl of PNK (NEB). The reactions were allowed to incubate at 37°C overnight, after which time, excess ATP was removed with Bio-Rad microfuge exchange spin columns. Samples were then lyophilized and extracted into 100 μl 10 mM TE buffer (10 mM Tris-HCl/1 mM EDTA, pH 7.2).

DNA was then treated with [Rh(bpy)2(phzi)]³⁺ or [Rh(bpy)2chrysi]³⁺ and irradiated. For each cell line, control samples, containing either metal complex without irradiation or irradiation in the absence of metal complex, were also prepared and analyzed. Samples being photolyzed were exposed to irradiation in the absence of metal complex, were also pre-

Fig. 1. Bulky metallointercalators. (Top) [Rh(bpy)2(phzi)]Cl₃. (Middle) The phi, chrysi, and phzi intercalating ligands. (Bottom) A view of intercalation of the Rh moiety (red) within a well matched DNA site (blue). The view of intercalation (based upon crystallographic coordinates; ref. 9) shows the snug fit of the phi ligand within its binding site. The bulky chrysi and phzi ligands are precluded sterically from intercalation in a similar fashion.

7.0, to 100% acetonitrile). NMR spectra were recorded on a Varian Mercury 300 MHz instrument. DNA synthesis was performed on an ABI 392 DNA/RNA synthesizer (Applied Biosystems) by using reagents from Glen Research. DNA was purified by using Glen Research PolyPak II cartridges (Glen Research, Sterling, VA), followed by reverse phase HPLC. Photocleavage reactions were carried out by using a 1,000-W Oriel Hg/Xe arc lamp (Oriel, Stamford, CT) with a mono-
to 440 nm light for 5 min. All samples were then lyophilized and extracted into 10 μl of 8 M urea loading dye and heated at 90°C for 2 min. The samples were then treated with 10 μl of 2× alkaline gel buffer and loaded on an alkaline denaturing 1% agarose gel and electrophoresed.

The gels were visualized and quantitated by using IMAGEQUANT software. Line integration down the center of the lanes was carried out to give a quantitative distribution of fragment intensity as a function of gel mobility. The distribution curves were then normalized by using ORIGIN. The dark control data were plotted for each cell line and fit to a Gaussian curve to obtain a midpoint. The raw, normalized intensity data up to the control midpoint for each cell line were then subtracted from the raw, normalized intensity data for the control. The resulting areas were then integrated to obtain relative values of shift in size distribution. These values were then averaged over several trials.

Results

Synthesis and Spectral Characterization of [Rh(bpy)2phzi]Cl3. [Rh(bpy)2phzi]Cl3 was prepared as illustrated in Fig. 2. The complex may be readily synthesized by using the condensation method (13). The benzo[a]phenazine quinone is made by the reaction of 2,3-dichloro-1,4-napthoquinone with o-phenylene-diamine in pyridine, followed by oxidation with nitric acid (12). Separately, [Rh(bpy)2(NH3)2](OTf)3 is synthesized (14), and then the condensation reaction is carried out in an H2O/acetonitrile solution-containing base. The reaction, as monitored by HPLC, is essentially complete after 30 min at ambient temperatures. The condensation method is significantly less cumbersome than reaction involving direct coordination of either the quinone diamine or diimine. Moreover, the reaction is also more facile than that with the chrysi quinone, given the lower extent of steric crowding around the condensation sites.

Spectral characterization for [Rh(bpy)2phzi]3+ resembles that for the parent phi and chrysi complexes. The spectrum consists of contributions from ligand to metal charge-transfer states near 310 nm and two ligand-centered transitions near 350 and 400 nm. Changes in the spectrum as a function of pH are consistent with deprotonation of the diimine at pH 5, similar to that for the chrysi complex.

DNA Photocleavage of a Single Base Mismatch. As is evident in Fig. 3, photoactivation of [Rh(bpy)2phzi]3+ bound to a mismatched site leads to direct strand cleavage. Upon photocleavage of [Rh(bpy)2phzi]3+ in the presence of a mismatch-containing DNA duplex, site-specific photocleavage is observed at the base 3’- to the mismatch site. Both the position of strand cleavage and the level of specificity are in accord with that seen with the chrysi complex. Significantly, however, specific cleavage of the mismatch site by [Rh(bpy)2phzi]3+ can be seen at metal concentrations as low as 10 nM.

Titrations as a function of concentration, where the ratio of metal/DNA is kept constant, can be used to establish the specific binding constant to an individual site. Fig. 4 shows the quantitation for such a photocleavage titration. The specific binding constant for [Rh(bpy)2phzi]3+ at the CC mismatch in this oligonucleotide is 1 × 107 M⁻¹. This compares to a value of 3 × 106 M⁻¹ for [Rh(bpy)2chrysi]3+ under comparable conditions.

Not only does [Rh(bpy)2phzi]3+ bind more tightly to mismatched sites, photoactivation experiments also reveal that the complex has approximately a fivefold higher photoefficiency than the chrysi analogue in cleaving the CC mismatch. The last three lanes in Fig. 3 also show the photoefficiency for the complex at different wavelengths. Remarkably, the cleavage can be seen even at visible wavelengths of 525 nm.

Site-specific binding by [Rh(bpy)2phzi]3+ can also be compared quantitatively to nonspecific binding to well matched sites on the duplex (Fig. 4). For this oligonucleotide, the highest cleavage intensity other than that for the CC site is evident within the duplex region 5’-OTCGG-3’. The determination of the binding constant for this site yielded a value of 4 × 10⁶ M⁻¹, almost two orders of magnitude weaker than that for the mismatch. The basis for reaction at this site is unclear. This site represents the highest well matched site affinity, so that the average nonspecific affinity is weaker. This level of specificity is comparable to that seen with the chrysi complex (4).

[Rh(bpy)2phzi]3+ targets not only the CC mismatch but other mismatches as well. The full family of mismatches was examined through photocleavage experiments on a 31-bp oligomer by varying the base sequence at the central mismatch site. The pyrimidine mismatches in particular, including CT, AG, and TA, are also readily cleaved. By quantitative photocleavage titration, the binding affinity for the CA mismatch is found to be 3 × 10⁶ M⁻¹, whereas that for CT is 6 × 10⁷ M⁻¹. Overall, targeting seems to be mismatch-specific, not base-specific. As with the parent chrysi complex, then, site-specificity depends on the local helix destabilization associated with mispairing.

Also of interest, whereas [Rh(bpy)2phzi]3+ has the ability to target a variety of different mismatches with high affinity, it is not able to cleave all mismatches with equal photoefficiency. This variation may be determined through quantitative photocleavage titration; at maximum binding, different intensities of photocleavage are observed. The different photoefficiencies can be understood in terms of variations in bound geometries within the different mismatch sites, because photocleavage is expected to depend on access of the photoexcited ligand radical to hydrogen atoms on the sugar (7). Several mismatches, such as the CC and TT, are cleaved with much better efficiency than with [Rh(bpy)2chrysi]3+: others, such as CA, are bound tightly but do not exhibit efficient cleavage.
Photocleavage of DNA from Cell Lines Proficient or Deficient in Mismatch Repair. As a test of the specificity and applicability of these complexes, photocleavage was also carried out on genomic DNA isolated from human cancer cell lines with or without compromised mismatch repair. Specifically, the HCT15, SW620, SKOV3, and DU145 cell lines were used. SW620 cells are fully competent in mismatch repair. HCT15 cells have an MSH6 mutation. SKOV3 and DU145 cells, containing mutations in MLH1 and both MLH1 and PMS2, respectively, are deficient in mismatch repair (2, 3, 16–19).

The genomic DNA was first digested into 5-kb lengths by restriction, radioactively end-labeled, treated with either [Rh(bpy)2chrysi]Cl3 (BC) and [Rh(bpy)2phzi]Cl3 (BZ) on 5'-32P-labeled (indicated by *) CC mismatch-containing 31/41-mer DNA duplex (1 μM). All samples were prepared in 10 mM Tris, pH 8.0/50 mM NaCl. All irradiations were at 313 nm for 15 min after DNA photocleavage by varying the concentration of [Rh(bpy)2chrysi]Cl3 (BC) and [Rh(bpy)2phzi]Cl3 (BZ), as well as at different wavelengths for [Rh(bpy)2phzi]Cl3. It is notable that BC does not cleave the DNA at concentrations up to 100 nM, whereas BZ already shows a cleavage at 10 nM at the mismatch site. AG and CT, Maxam-Gilbert sequencing reactions; DC, dark control DNA sample without irradiation; LC, the light control sample irradiated in the absence of metal complex.

Significant cleavage is evident with [Rh(bpy)2phzi]3+ at 50 nM concentration for DNA from SKOV3 and DU145, the two cell lines that lack MLH1. A low level of photocleavage is apparent with HCT15, lacking MSH6, and no significant cleavage over the controls is apparent with SW620, a cell line competent in mismatch repair. The photocleavage results correlate directly with independent analyses of these cell lines for microsatellite instabilities (16–19).

Similar trends are evident for the chrysi complex, although higher concentrations of the metal complex must be used and only a small differential effect is apparent. Interestingly, the highest level of discrimination in cleavage among the different cell lines was found at 0.1 nM [Rh(bpy)2phzi]3+.

**Discussion**

**Design of a High-Affinity Reagent Targeted to Mismatches.** A mismatch recognition agent has been synthesized with a hetero-
cyclic aromatic ligand system based on the condensation technique. As with the parent rhodium intercalators, [Rh(bpy)2phzi]3+ promotes direct strand cleavage at its target site with photoactivation. Importantly, photocleavage experiments using [Rh(bpy)2phzi]3+ show that this intercalator can recognize DNA mismatches with very high specificity at concentrations in the nanomolar range.

The fact that this bulky intercalator, like the parent chrysi complex, is able to specifically target mismatches suggests the generality of this targeting strategy. By increasing the expanse of the intercalating ligand beyond that of a base pair, binding to well matched DNA sites is disfavored owing to shape.
means to distinguish among these different cell lines based on their mismatch repair activity.

Targeting and discrimination among different genomic DNAs, therefore, can be considered based on the increased frequency of mispairing in genomic DNA from mismatch repair deficient cells. Because the metal complexes are specific in targeting mismatches, their levels of photocleavage should reflect the levels of mismatches accumulated. The heterogeneity in fragment length both before and after photocleavage makes absolute quantitation of the frequency of mismatches in these cell lines difficult. Nonetheless, differential DNA cleavage is evident among these cell lines with nanomolar concentrations of the metal complex. It is also noteworthy that the results described thus far reflect overall mismatch accumulation in the genome. By probing individual genes through photocleavage, gene-specific analyses could also be envisioned.

Implications for Therapeutic Design. Whereas chemotherapy in the past has focused largely on applications of cytotoxic agents lacking selectivity, current strategies are directed at developing means to discriminate and selectively target cancerous cells. Discrimination based on mismatch recognition offers the opportunity for such selective targeting. Defects in the mismatch repair system are commonly associated with different cancers (2, 16). By specifically targeting the genomic DNA of such cancerous cells with small molecules that recognize and generate lesions in mismatch-containing DNA, preferential damage to cancerous cells could be generated. The design and application of bulky metallointercalators that bind mismatches with high affinity, as described here, represent a foundation for such strategies. Complexes have been designed with high selectivity for mismatches and with high affinity. Moreover, the differential targeting of genomic DNA from cell lines based on their compromised mismatch repair systems has been demonstrated. Creating new generations of complexes with applicability in vivo remains a challenge, but the diversity in structure afforded by octahedral metallointercalators should make such design viable.

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