Studies of the Interaction of Platinum Drugs with DNA Using Oligonucleotide Microarrays

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Summary: A novel method for the study of the interaction of the platinum drug cis-diamminedichloroplatinum(II) (cis-DDP or cisplatin) with 50-mer oligonucleotides that were printed in high throughput microarray format is introduced. Our aim has been to identify sequence level differences in the interaction of various drug candidates that may serve to enable rational targeting of drugs to specific genes. A microarray of 26 control genes commonly used in oligonucleotide, Affymetrix and c-DNA microarray platforms were microcontact spotted as amine-terminated 50-mer oligonucleotides onto glycidoxypropyltimethoxy silane (GPMS)-modified glass slides. The generalized study format involved hybridization of probes with 10 fluorescently labeled complements as target followed by confocal imaging to reveal original spot intensities. Microarrays were then incubated at 37 °C with hydrolysed cisplatin while in hybridization cassettes, washed in buffer and then scanned again to reveal secondary intensities. We have investigated the influence of cisplatin to stabilize the relative fluorescence intensity via intrastrand crosslinking by studying the impact of varying drug:probe-DNA mole ratio (0:1 (blank), 1:1, 25:1 and 50:1) and annealing temperatures (36, 46, or 56 °C) on retained intensity. ANOVA revealed that 4 of the 10 genes demonstrated ($p < 0.0001$) the expected result of increased signal retention with decreased temperature and increased drug concentration.

Keywords: cisplatin; DNA; drugs; microarrays; oligonucleotides

Introduction

The platinum drug cisplatin (*cis*-diamminedichloroplatinum(II) *cis*-$[PtCl_2(NH_3)_2]$ has been shown to be a powerful chemotherapeutic agent in the effective treatment for a variety of cancers, particularly testicular cancer, head and neck carcinoma and lung carcinoma[1]. The primary target of cisplatin is celluar DNA[2]. The interaction of this drug with DNA has been the subject of considerable research[2–4]. The effectiveness of cisplatin as a chemotherapeutic agent has been proposed to result from interstrand and intrastrand crosslinks that interfere with replication and transcription of DNA, as well as mechanisms of DNA repair, and as a consequence, results in apoptosis[5]. Under cellular conditions, a single molecule of water replaces one of the chloride ions (viz. *cis*-$[PtCl(NH_3)_2(H_2O)]^+$) resulting in a perpensity of the aquated cisplatin to bind to a single nitrogen on a DNA nucleotide. Subsequently, the second chloride ion is replaced by a second H_2O molecule and the platinum binds to a second nucleotide. Binding studies have indicated a preference for nitrogen 7 on two adjacent guanines on the same strand (1,2-intrastrand adducts) but also binding to adenine and across strands

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(1,2-interstrand adducts) to a lesser extent [6]. The conformationaly distorted cisplatin-DNA complex irreversibly binds HMG (high mobility group)-1 and other DNA repair proteins, preventing effective repair of the DNA strand. The intrastrand and interstrand cross-links of cisplatin with cellular DNA have been linked to the overall cytotoxicity of cisplatin resulting in an ID_{50} (L1210) of 2.4 mM and TWI%(LX-1) of 38 @ 4.0 mg/kg[7].

The interaction of cisplatin with DNA has been examined by electrophoretic techniques, ^{1}H NMR[8] and by anolmalous scattering[4]. Here we propose and demonstrate for the first time the novel use of DNA microarrays for the direct study of drug-DNA interactions. DNA microarrays represent a unique platform for the high throughput examination of such interactions. Microarrays have primarily been used to examine gene expression profiles for disease classification and functional genomics[9] and their value in drug development remains largely under explored[10]. DNA microarrays allow direct detection of interaction between a drug molecule and varied templates of DNA. DNA microarrays also have the additional advantage that thousands of genes may be tested simultaneously, allowing gene-specific interactions to be determined. Gene specificity for cisplatin has been proposed to result from electrostatic interactions and hydrogen bonding of cisplatin before covalent attachment[10].

Experimental

Materials

DNA Probes

Printed 50-base 5′ C_6-NH_2-modified oligonucleotide DNA probes were chosen from a list of common housekeeping genes and several calibration features found on the Affymetrix (U133) gene chip and the C3B 10K Human Oligogonucleotide Microarray[11]. These 25 genes and their sequences are shown in Table 1 and were synthesized and purified by MWG Biotech, supplied as lyophilized pellets, and resuspended in DI water.

Oligonucleotide Arrays

DNA microarrays were prepared by microcontact printing (μCP) using 6 micromachined silicon pins per print head (Parallel Synthesis Technologies, Inc.) to transfer the 25 amine-terminated oligonucleotides of Table 1 onto glycidoxyproplytrimethoxy silane (GPS) (Sigma Aldrich, # 44016-7) modified glass microscope slides (Gold Seal, # 3010)[11]. Use was made of a Cartesian PixSys 4500 microarrayer operating under 50–55%RH and 25+/− °C. All processing were done under class 1000 clean room conditions. The spotting buffer consisted of 1.5 M betaine (Sigma Aldrich # B2629), 3X SSC and 25 μM oligonucleotide. The gene probes were spotted into microarrays comprising 2 replicates of 6 (5×9) sub-arrays. Within each replicate, the first three sub-arrays comprised columns of five replicates of each of 9 genes and each column was replicated by randomizing the gene-column order and spotting a second sub-array adjacent to the first. In this way each gene was spotted 20 times on each microarray as shown in Figure 1. Post-printing, the microarrays were cured at 40 °C and 50% RH for 48 hours, washed in 0.2% sodium dodecyl sulfate (SDS) solution, rinsed in ultrapure H_2O, and incubated in 50 °C ultrapure H_2O for 20 min to remove excess DNA, followed by storage in the dark under dessicator conditions until use.

DNA Targets

Labeled oligonucleotide targets were synthesized by MWG Biotech as 50-base 5′-C_6-Cyanine 3-modified oligonucleotides and were complimentary to ten (10) of the 26 gene sequences immobilized on the microarray. These 10 labeled target sequences included five human genes, 2 *E. coli* genes, and 3 *B. subtilis* genes and are identified in Table 1 as the shaded members of the list.

Table 1.
Probe annotation and sequences used in constructing the control gene array. Green letters indicate human derived genes, and Blue letters indicate bacteria derived genes.

Accession	Gene Name	Probe Sequence	% GC
M64784 *	phosphofructokinase, platelet	tcccaaagaacagtggtggctcaagctacggcccctcatgaaaatcctgg	54
M27396	asparagine synthetase	aaagaaggatattactaccgtcaagtctttgaacgccattacccaggccg	
M11560	aldolase A, fructose-bisphosphate	ccctcagccctcgccatcatggaaaatgccaatgttctggcccgttatgc	
XM_083842 ◆	phosphoglycerate mutase 1 (brain)	gcgaagcgcggcgggcaggcgctacgagatgctggctatgagtttgacat	62
NM_000291	phosphoglycerate kinase 1	atgctgaggctgtcactcgggctaagcagattgtgtggaatggtcctgtg	54
NM_014763 +	mitochondrial ribosomal protein L19	gatatgatgagggaatatgatacttcaaaaattgaagctgcaatatggaa	32
AA453756	Rho GDP dissociation inhibitor (GDI) alpha	cttttgtactagctgtctccatattatgttcaataaattctgtgctctga	
NM_005566	lactate dehydrogenase A	gactctgacttctgaggaagaggcccgtttgaagaagagtgcagatacac	
M12996	glucose-6-phosphate dehydrogenase	tgacctacggcaacagatacaagaacgtgaagctccctgagccctacgag	
AB061838 •	Ribosomal protein S3	tgactgctgtagttcagaagaggtttggctttccagagggcagtgtagag	50
XM_088688	non-POU-domain-containing, octamer-binding	aatatggaaaggcaggcgaagtcttcattcataaggataaaggatttggc	
NM_002954 •	Ribosomal protein S27a	tgcccttctgatgaatgtggtgctggggtgtttatggcaagtcactttga	48
NM_004048	beta-2-microglobulin	tggagagagaattgaaaaagtggagcattcagacttgtctttcagcaagg	
X00351	3′ mRNA for beta-actin	agcaagcaggagtatgacgagtccggcccctccatcgtccaccgcaaatg	
X00351	5′ mRNa for beta-actin	caactgggacgacatggagaaaatctggcaccacaccttctacaatgagc	
M33197	3′ Glyceraldehyde-3-phosphate dehydrogenase	tctgacttcaacagcgacacccactcctccacctttgacgctggggctgg	
M33197	5′ Glyceraldehyde-3-phosphate dehydrogenase	tgcttttaactctggtaaagtggatattgttgccatcaatgaccccttca	
M97935	3′ Homo sapiens transcription factor ISGF	atccaaatattgacaaagaccatgcctttggaaagtattactccaggcca	
M97936	5′ Homo sapiens transcription factor ISGF	acaaaaaacaaacaagtgttatgggaccgcaccttcagtcttttccagca	
J04422 ■	E. coli bioB gene biotin synthetase	gcaactgttccgcaaactggggctaaatccgcagcaaactgccgtgctgg	58
J04423 ▲	E. coli bioC protein	atcacgctgtggtttgatgatgcgctcagtgccatgcgttcgctgaaagg	54
J04423	E coli bioD gene dethiobiotin synthetase	acgctgaatatatgaccacgctcacccgcatgattcccgcgccgctgctg	
X04603 -	B subtilis thrC, thrB gene	ggcgcatatggaacggctctcagcggagcaggcccaacgattctcgtcat	60
M24537 ×	B subtilis pheB, pheA genes	tcacgtccgactaaaaccggattaggccattatttctttattattgatat	34
X17013 —	B subtilis lys gene for diaminopimelate	gcgtccctctctagctaaaagagatgcacgatcaatggcctgaaggaatt	48

Buffers and Reagents

The hybridization/wash buffer was prepared from 0.03 M Na_2HPO_4, 0.125X SSC (sodium chloride, sodium citrate), 0.125 mM EDTA (ethylenediaminetetraacetic acid), 0.25X Denhardts Reagent (Ficoll Type 400, polyvinylpyrrolidone, BSA), 0.06% SDS (~100 mM Na^+) and was used for both the target – probe hybridization and subsequent washing steps. The Cy3-labeled target DNA mixture was prepared in hybridization/wash buffer at ~500 pM in each of the ten target oligonucleotides. Cisplatin (0.70 mM) was prepared in 1.76 mM $AgNO_3$, filtered (0.22 μ) and proportionately combined with incubation buffer to achieve the final challenge concentration.

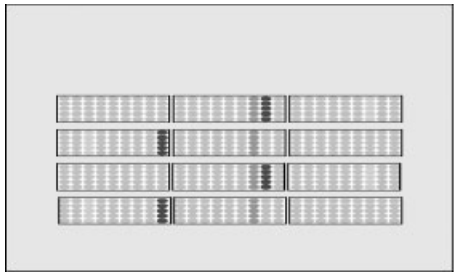

Figure 1.
Spotted oligonucleotide microarray showing 12 5x9 sub arrays.

Methods and Procedures

Experimental Design

The drug incubation and subsequent temperature-controlled washing steps were established as a 3^2 factorial design with three incubation temperatures (36, 46, or 56 °C) and three drug concentrations (1:1, 25:1 and 50:1). The effect of ''drug'', ''temperature'' and ''drug-temperature interaction'' was evaluated through the response variable, retained signal fluorescent intensity, i.e. the difference between SCAN 1 and SCAN 2.

Hybridizatons

Hybridization incubation was preformed in ArrayIt® hybridization cassettes (Telechem Int., AHC). The target-probe hybridization reaction was performed by securing a lifter cover slip (M-5439-0001-LS, Erie Scientific) to the microarray by wrapping with parafilm (PM-999 Pechiney Plastic Packaging). A hybridization cocktail consisting of 35 μL hybridization buffer, 1 μL 100X spike-in control solution, and 34 μL ultra pure H_2O was pipetted beneath the lifter cover slip onto each microarray. A hydration solution of 100 μL ultra pure H_2O was dispensed into the recesses at the bottom of each well and the cassette was covered and sealed. The cassette assembly was then placed into a convection oven at 56+/1 °C for 16 hr. Following hybridization incubation, microarrays were washed in hybridization buffer at room temperature (~24 °C) for 10 min then rinsed three times in ultra pure H_2O before being spun dry at 1,300 rpm for 3 minutes in a centrifuge (Eppendorf, 5804 R). The microarrays were then scanned using a ScanArray Express (Perkin Elmer) confocal laser scanner (80% laser power and 80% gain) and images were saved for quantification using Quantarray software (SCAN 1). Following scanning, the microarrays were immediately set up for incubation with cisplatin using the method described above.

Drug Incubations

Cisplatin solutions were applied at varying concentrations specified by molar ratio of Drug:DNA of 0:1 (control), 1:1 (0.029 μM), 25:1 (0.714 μM), and 50:1 (1.429 μM). The microarrays were similarly incubated within the hybridization cassette and within the oven at 37 °C for two hours to permit drug-DNA adduct formation. Following incubation, the microarrays were placed in slide carriers and the three sets of four microarrays were then submerged in wash buffer for 10 minutes at wash temperatures 36 °C, 46 °C, or 56 °C. Following the wash, the microarrays were rinsed in ultra pure H_2O and spun at 1,300 rpm for 3 minutes to dry. The arrays were scanned again and the images were quantified using Quantarray. The microarrays were similarly scanned and the images were saved for quantification using Quantarray software (SCAN 1).

Data Analysis

The resulting data was modeled within SAS and the ANOVA model [1], wherein

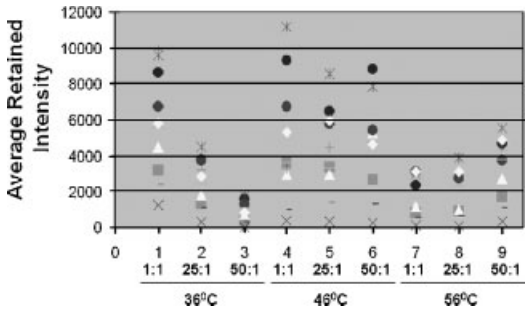

Figure 2.
Plots of average retained signal intensity (SCAN 1–SCAN 2) for each gene. Test conditions (arrays) are organized by temperature and then by drug concentration. Symbols in Table 1.

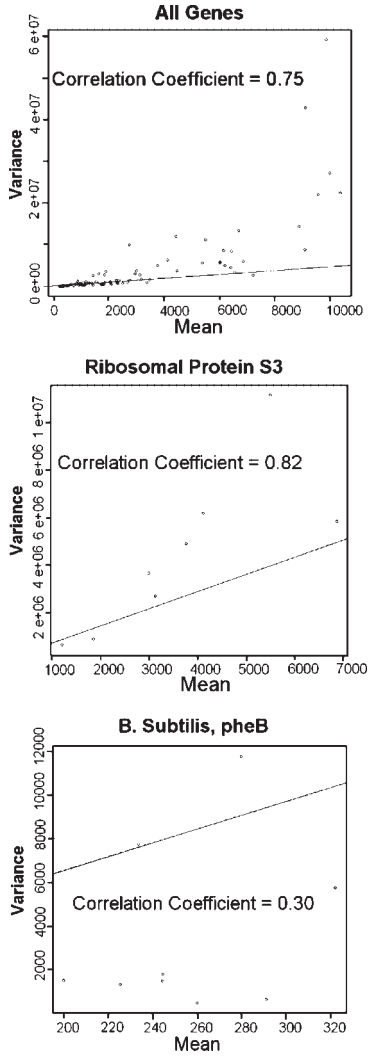

Figure 3.
Example ''Mean by Variance'' plots for; all genes, for ribosomal protein s3, and for *B. Subtilis* Phe B. The correlation coefficient is displayed on each plot. The left-most plot is of all genes and the high correlation coefficient indicates heteroscedasticy in the complete data set. The middle plot is for a single gene which also displays a non-random distribution. The far right displays a more random distribution of points and a low R^2 value indicating that the intensity associated with this probe may be tested for significance between experimental conditions.

temperature and drug concentration were specified as fixed effects.

$$y_{ijkgr} = \mu + A_i + T_j + D_k + TD_{jk} + \varepsilon_{ijkgr} \quad (1)$$

Where the levels of temperature (T) ranged from i = 1–3, and the levels of drug concentration (D) ranged from j = 1–3. The temperature – drug concentration interaction was denoted by TD. The effect of arrays (A) was also included in the model where i ranges from 1–9 are r denotes replicate spots for each gene (g) 1–10.

Results and Discussion

This experiment was set up and conducted to test the hypothesis that cisplatin would, via 1,2-intrastrand adduct formation, act to stabilize hybridized DNA and effectively increase its melting temperature and that increasing amounts of cisplatin should likewise effectively increase melting temperature. For any given gene (the average intensity of spots arranged in a row and replicated throughout the 12 sub-arrays), it is anticipated that those arrays that were washed at the lowest temperature (36 °C) and highest drug concentration (50:1) should have the highest retained intensity (SCAN 1–SCAN 2). Such an observation would be the result of uniform 1,2-intrastrand adduct formation across all genes over all arrays. Figure 2 shows the actual results obtained.

However, 1,2-intrastrand adduct formation is not expected to be uniform throughout the array. Analysis of variance (ANOVA) was performed on pooled spot intensity values from select genes taken from all arrays. ANOVA could not be preformed on pooled intensity values from all genes due to heteroscedastic trends in the data. However, since not every gene showed heteroscedasticy, some gene-by-gene analysis was possible. A ''Mean by Variance'' plot, which in the absence of heteroscedasticy is expected to display no correlation, was produced for each gene to check for heteroscedacticity and the model [1] was applied to each gene that passed the arbitrarily assigned R^2 criteria of $R^2 < 0.45$. Figure 3 illustrates this approach.

The results revealed a significant effect due to drug, temperature and the drug-

Table 2.
Significance of main effects and interaction per gene, $p < 0.0001$. F-test values are given for the fixed effect Model 1, dashed lines indicate that ANOVA was not performed due to detected heteroscedacticity.

Gene	GC	Drug F-Test	Temp F-Test	Drug*Temp F-Test
phosphoglycerate mutase 1 (brain)	62	23.32	76.67	40.85
phosphofructokinase, platelet	54	----	----	----
mitochondrial ribosomal protein L19	32	178.17	116.20	22.77
ribosomal protein S3	50	----	----	----
ribosomal protein S27a	48	----	----	----
E. coli bioB gene biotin synthetase	58	----	----	----
E. coli bioC protein	54	----	----	----
B subtilis thrC, thrB gene	60	----	----	----
B subtilis pheB, pheA genes	34	2.86	26.75	7.92
B subtilis lys gene for diaminopimelate	48	13.284	136.86	42.89

temperature interaction for several genes and these results of F-testing are summarized in Table 2. Thus, a significant stabilizing effect due to inclusion of the drug was detected for particular genes. Furthermore, the higher the concentration of drug added the greater the stabilizing effect observed.

Conclusions

A novel method for the study of cisplatin-DNA interaction in high throughput format is presented. Microarrays of immobilized oligonucleotides afford a convenient format to study drug-DNA interactions. This may be accomplished by incubating the drug with the surface-confined probe or with the solution-borne labeled target (ssDNA) prior to hybridization. Also, this method may be used to rapidly investigate the binding and or covalent interactions of novel di-nuclear and tri-nuclear platinum compounds[12] that may be candidates for drug development.

References and Notes

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