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Research Article

A Qualitative and Quantitative Assay to Study DNA/Drug Interaction Based on Sequence Selective Inhibition of Restriction Endonucleases

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Abstract

Purpose: *To explore the use of restriction inhibition assay (RIA) to study the binding specificity of some anticancer drugs.*

Methods: *A 448 bp DNA fragment derived from pBCKS+ plasmid (harboring the polylinker region with multiple restriction endonuclease sites) was used as a template for sequence selective inhibition of the test drugs. The template was incubated with different concentrations of anticancer drugs (adriamycin, daunomycin, mitoxantrone, distamycin-A, berberine and palmatine) prior to digestion with restriction endonucleases - Hin*dIII, *Eco*RI and *Eco*RV.

Results: *Mitoxantrone, adriamycin and daunomycin showed specificity for Hin*d*III restriction site (5'-AAGCTT-3') at 220, 100 and 100 µM concentration, respectively. Conversely, distamycin-A showed an affinity for Eco*RI *(5'-AAATGC-3') restriction sites at a concentration of 10 µM. No binding was observed for berberine and palmatine at a maximum concentration of 2 mM at Hin*dIII, *Eco*RI *and Eco*RV *restriction sites, respectively.*

Conclusion: *The inhibition of endonucleases by mitoxantrone, adriamycin, daunomycin, distamycin-A, provides direct evidence of the co-existence of concentration and sequence specificity for drug-DNA interaction as well as the need to explore the possible use of RIA for demonstrating the binding specificity of anticancer drugs.*

Keywords: *Restriction endonucleases, Restriction sites, Anticancer drugs, Restriction inhibition assay (RIA), Binding specificity.*

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INTRODUCTION

Interaction of some anti-tumor agents with DNA has been the subject of intensive research. In this regard, considerable amount of effort has been made to elucidate the molecular basis of action of several drugs such as adriamycin, daunomycin, distamycin-A, mitoxantrone, berberine and palmatine, which are known to bind to DNA. These drugs have good anti-tumor activity and this activity has been correlated to sequence specific binding to DNA. Sequence specificity of these drugs has been attempted using DNA foot printing, *in vitro* transcription assay, restriction inhibition assay, absorption spectroscopy, fluorescence, circular dichroism spectroscopy, Nuclear Magnetic Resonance (NMR) spectroscopy and X ray crystallography. DNase foot printing experiments have shown preference for CpG, CpA, TpA sites in DNA with mitoxantrone [1]. Adriamycin and daunomycin have shown affinity for CpA rich sequences [2]. Transcriptional inhibition assay have shown preferences for 5'-(A/T) CA and 5'-(A/T)CG sites on DNA for mitoxantrone [3] and 5'-TCA for adriamycin and GC flanked at 5' by AT sequences for daunomycin [4]. In this context restriction inhibition assay showed more specificity for CA- than GC-rich sequence for adriamycin and more affinity towards GC than AT rich sequence for daunomycin [5], distamycin-A showed more specificity for AT rich sequence [6].

The structures of some drugs complexed with specific oligonucleotide have been studied in recent times by X-ray and NMR. The structure of adriamycin complexed with DNA and studied by X-ray [7] and NMR [8] have shown that in these complexes the chromophore is intercalated at CpG, while in the case of daunomycin [9,10]], it was noticed that daunomycin intercalates in the d(CpG) and d(TpG) sequences respectively. Structural studies of distamycin A with sequences such as d $(CGCGAATTCGCG)_2$ [11] and d$(CGCAAATTTGCG)_2$ using NMR [12] and X-ray [13] respectively, have shown that distamycin-A prefers AT-rich sequence in the minor groove of DNA. In this context, we embarked on experiments to detect the sequence specificity of berberine, palmatine and mitoxantrone whose sequence specific interaction with *Hin*dIII (5'-AAGCTT-3'), *Eco*RI (5'-AAATGC-3') and *Eco*RV (5'-AATAGC-3') binding sites remained to be explored by restriction inhibition assay. We have also confirmed daunomycin, adriamycin and distamycin-Sequence specificity using Restriction Inhibition Assay (RIA) in conjunction with data from available literature was adopted in which the protocol was standardized the protocol and used for our experimental work.

EXPERIMENTAL

Drugs and chemicals

Adriamycin, daunomycin, distamycin-A, mitoxantrone, berberine and palmatine were purchased from Sigma Aldrich Co. USA. Restriction endonucleases *Pvu*II, *Hin*dIII, *Eco*RI and *Eco*RV supplied with NEB (10 x) restriction buffer (100 mM Tris-HCl pH 7.5, 1 mM EDTA, 100 mM magnesium acetate, 500 mM potassium acetate) were purchased from New England Biolab, UK. Stock solutions of distamycin-A (100 µM), mitoxantrone (1mM), adriamycin (100 mM), daunomycin (1mM), berberine (100 mM) and palmatine (100 mM) were prepared by dissolving each initially in minimum autoclaved milli-Q water before making up to volume with the same. All solutions were stored at 4 °C.

DNA fragment for restriction inhibition assay (RIA)

The 448 bp fragment was obtained from $pBCKS^+$ DNA by the following procedure. The $pBCKS^+$ DNA was transformed into *E. coli*. Cell bearing plasmids were grown and amplified with chloramphenicol (20 µg/ml), and the DNA was isolated and purified by alkaline lysis method [14]. The $pBCKS^+$ plasmid DNA samples were stored at -20 °C in Tris HCl buffer (10 mM Tris–HCl, pH 8.0) containing 1 mM EDTA. The 448 bp DNA

fragment was derived from pBCKS+ plasmid (harboring the polylinker region with multiple restriction endonuclease sites) by restricting with *Pvu*II restriction enzyme and eluting the 448 bp fragment by gel elution and then purifying it further by phenol, chloroform and isoamyl alcohol method and ethanol precipitation. The 448 bp fragment was stored at -20 °C in Tris HCl buffer (10 mM Tris HCl, pH 8.0) containing 1 mM EDTA.

Binding studies

The anti-tumor drug–DNA complexes were prepared by preincubating 4 µl (300 ng) of the 448 bp DNA fragment with a range of concentration of the drugs in NEB restriction buffer (1 x) for 30 min at 37 °C. The restriction digestion was carried out by incubating with *Eco*RI, *Eco*RV and *Hin*dIII respectively for a further 1 h at 37 °C in a final volume of 20 µl. A control setup with the DNA along with each restriction enzyme alone was kept to analyze the results of the restriction inhibition assay. Each digestion was stopped by incubation for 20 min at 65 °C followed by 10 min at 4 °C. A 20 µl of the digestion mixture was added with 5 µl of 6 x DNA loading dye (0.25 % bromophenol blue and 30 % glycerol) and loaded on to 2 % horizontal agarose gel in case of 448 bp DNA fragment which was run in Tris-acetate EDTA buffer (40 mM Tris base, pH 8.0, 18 mM glacial acetic acid and 1 mM EDTA) at 100 V for 2 h. The gel was exposed to 220 nm near UV region spectrum and then the DNA bands were visualized and analyzed for drug-DNA interaction studies.

RESULTS

Binding of distamycin-A, adriamycin and daunomycin to the *Eco*RI (5'-AAATGC-3') and *Hin*dIII (5'-AAGCTT-3') restriction sequence

The results on the detection of binding of Distamycin-A, Adriamycin and Daunomycin to DNA by restriction inhibition assay (RIA).are shown in Fig 1 - 3. We first produced a 448 bp target double stranded DNA fragment consisting of the polylinker region from the pBCKS$^+$ plasmid DNA. Second, we allowed distamycin-A, adriamycin and daunomycin to bind to the 448 bp double stranded DNA.

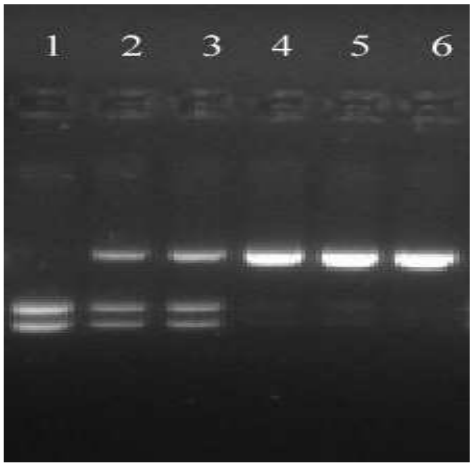

Figure 1: Agarose gel (2 %) showing inhibition of *Eco*RI cleavage of 448 bp fragments of pBCKS$^+$ by distamycin A. Lane 1: Control complete digest of 448 bp DNA fragment by *Eco*RI; Lanes 2-7: distamycin A-448 bp complex digested by *Eco*RI with distamycin-A concentrations of 2, 4, 6, 8, 10 and 12 µM, respectively.

In Fig 1, the binding of distamycin-A to the target, 448 bp double stranded DNA, was studied at different drug concentrations ranging from 2 to 12 µM. The experiment shows a gradual decrease in intensity of digested fragment in a concentration dependent manner which reaches complete inhibition of *Eco*RI at 10 µM concentration by distamycin-A, showing specificity for AT sequence. Similarly, Figs 2 and 3 demonstrate that adriamycin and daunomycin studied at different drug concentrations inhibited the restriction activity of *Hin*dIII in a concentration dependent manner and showed specificity for GC containing sequences. The experimental results demonstrate that both adriamycin and daunomycin show complete inhibition of *Hin*dIII at 100 µM concentration.

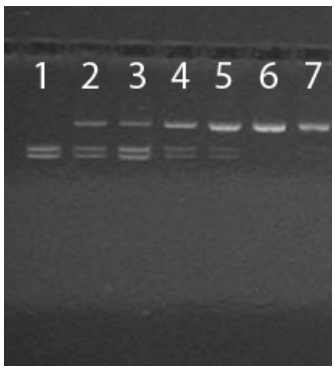

Figure 2: Agarose gel (2 %) showing inhibition of *Hin*dIII cleavage of 448 bp fragments of pBCKS$^+$ by adriamycin. Lane 1: Control complete digest of 448 bp DNA fragment by *Hin*dIII; Lanes 2-6: adriamycin-448 bp complex digested by *Eco*RI with adriamycin concentrations of 20, 40, 60, 80 and 100 µM, respectively.

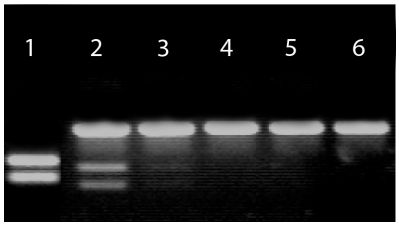

Figure 3: Agarose gel (2 %) showing inhibition of *Hin*dIII cleavage of 448 bp fragments of pBCKS$^+$ by daunomycin. Lane 1: Control complete digest of 448 bp DNA fragment by *Hin*dIII; Lanes 2-6: daunomycin-448 bp complex digested by *Hin*dIII with daunomycin concentrations of 80, 90, 100, 110 and 120 µM, respectively.

Detection of binding of mitoxantrone to the *Hin*dIII restriction sequence (5'-AAGCTT-3').

To determine whether binding of mitoxantrone to DNA is detectable by restriction inhibition assay, the experiment shown in Figures 1-3 was repeated using mitoxantone. Figure 4 shows the experiment conducted to demonstrate the binding specificity of mitoxantrone for *Hin*dIII restriction sequence (5'-AAGCTT-3'). The drug was studied at different concentrations ranging from 200 to 240 µM. An inhibition of *Hin*dIII, in a concentration dependent manner was observed for mitoxantrone and eventually at 220 µM a complete inhibition was seen. The result clearly shows sequence specific interaction of mitoxantrone with *Hin*dIII restriction sequence, 5'-AAGCTT-3'.

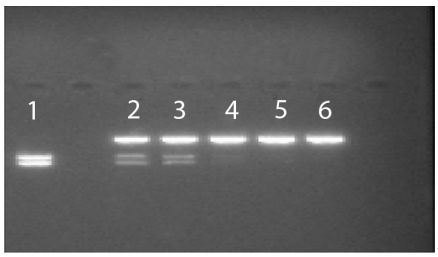

Figure 4: Agarose gel (2 %) showing inhibition of *Hin*dIII cleavage of 448 bp fragments of pBCKS$^+$ by mitoxantrone. Lane 1: Control complete digest of 448 bp DNA fragment by *Hin*dIII; Lanes 2-6: mitoxantrone-448 bp complex digested by *Hin*dIII with mitoxantrone concentrations of 200, 210, 220, 230 and 240 µM, respectively.

Detecting binding of palmatine and berberine to the *Eco*RI (5'-AAATGC-3') and *Eco*RV (5'-AATAGC-3') restriction sequences.

The assay was carried to study the binding specificity of berberine and palmatine. Figure 7 shows that under these experimental conditions, berberine and palmatine did not bind to both *Eco*RI (5'-AAGCTT-3') and *Eco*RV (5'-AATAGC-3') restriction sequences. The study was carried out using drugs at different concentrations, but even at 2 mM concentration no inhibition of both *Eco*RI and *Eco*RV was observed.

DISCUSSION

The principle of RIA assay is based upon the ability of different anti-tumor drugs to inhibit the cleavage activity of restriction endonucleases *Hin*dIII, *Eco*RI and *Eco*RV. In the gel assays of distamycin-A, adriamycin and daunomycin, a gradual decrease in the intensity of the digested fragment was observed and finally a 100 % inhibition of restriction was observed. It was observed that distamycin inhibits the restriction digestion of *Eco*RI at 10 µM concentration

and shows its sequence specificity towards AT site present in *Eco*RI. This result is in accordance with earlier studies [6]. They suggested that distamycin binds to AT rich sequences using restriction inhibition assay at 100 µM. It was then further proved by structural studies that distamycin binds to AT base pair by minor groove binding [11-13].

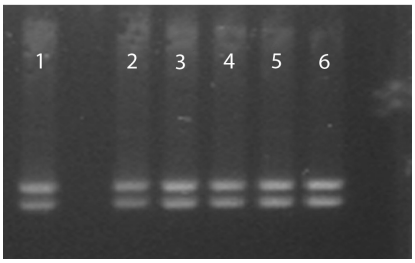

Figure 5: Agarose gel (2 %) showing inhibition of *EcoRI and EcoRV* cleavage of 448 bp fragments of pBCKS$^+$ by berberine and palmatine. Lane 1: Control complete digest of 448 bp DNA fragment by *Eco*RI; Lane 2: berberine-448 bp complex digested by *Eco*RI with berberine concentration of 2 mM; Lane 3: palmatine-448bp fragment complexes digested by *Eco*RI with palmatine concentration of 2 mM; Lane 4: Control complete digest of 448 bp DNA fragment by *Eco*RV; Lane 5: Berberine-448 bp fragment complexes digested by *Eco*RV with berberine concentration of 2 mM; Lane 6: palmatine-448 bp fragment complexes digested by *Eco*RV with palmatine concentration of 2 mM.

Adriamycin has been shown to bind to CA with higher affinity than GC rich sequences [2-5], using DNase foot printing assay, transcriptional and restriction inhibition assay. In our study adriamycin shows a complete inhibition for *Hin*dIII activity at 100 µM concentration and binds to GC rich sequences, which supports the sequence and structural studies done by a few authors using x-ray [7] and NMR [9] methods and this shows that it intercalates between CG base pair. Our results also support daunomycin affinity for GC containing sequence by 100 % inhibition of *Hin*dIII activity at 100 µM concentration. This result is in accordance with binding studies of daunomycin by foot printing and restriction inhibition assay [5], which showed preference of daunomycin for GC base pair flanked by AT base pair. Therefore RIA can provide sequence specificity for these standard drugs used in this study. Structural studies of daunomycin complexed with DNA using x-ray method showed that it intercalates between CG base pair [8]. There are no conclusive reports available regarding structure and conformation of mitoxantrone complexed to DNA. Therefore there is a need for the sequence specificity of mitoxantrone, berberine and palmatine. In this context Fig 4, shows a typical restriction inhibition assay gel for mitoxantrone including a control-the 448 bp DNA fragment without drug. As the concentration of mitoxantrone was increased (lanes 2-6) the intensity of the digested fragments decreased gradually.

Finally, 100 % inhibition in restriction was achieved at 220 µm (lane 4), when compared to the control (lane 1). This result throws an insight on the mitoxantrone specificity for 5'-GC-3'. This result supports the studies by Fox *et al* [1] and Panousis *et al* [3] using *in vitro* transcription assay and DNase foot printing assay, respectively. These assays show sequence specificity towards CG, CA and TA (reading in the 5'-3' direction). Figure 5 shows RIA assay gel of berberine and palmatine with *Eco*RI and *Eco*RV. Both palmatine and berberine did not demonstrate binding affinities for either *Eco*RI or *Eco*RV recognition sequences irrespective of their concentration. Experimentally standardized binding specificity and the binding concentration of distamycin-A, adriamycin, daunomycin, mitoxantrone, berberine and palmatine with the 448 bp DNA fragment are summarized in Table 1.

The specificity for each of the drugs was confirmed by performing a negative control experiment using *Eco*RI as negative control for drugs like mitoxantrone, adriamycin and daunomycin which bind to *Hin*dIII binding sites, and *Hin*dIII as control for drugs like distamycin-A, berberine and palmatine which are known to bind to either 5'-AT-3' or 5'-TA-3' containing sequences [data not shown].

Table 1: A summary of quantitative and qualitative binding data for the anticancer drugs

Drug	*DNA fragment length*	*Binding concentrations#*	*Drugs sequence specificity identified**	*Restriction enzyme used*	*Restriction enzyme restriction site*
Mitoxantrone	448 bp	220 mM	5'-AAGCTT-3'	*Hin*dIII	5'-AAGCTT-3'
Adriamycin	448 bp	100 µM	5'-AAGCTT-3'	*Hin*dIII	5'-AAGCTT-3'
Daunomycin	448 bp	100 µM	5'-AAGCTT-3'	*Hin*dIII	5'-AAGCTT-3'
Distamycin A	448 bp	10 mM	5'-GAATTC-3'	*Eco*RI	5'-GAATTC-3'
Berberine	448 bp	No Binding observed at 2 mM	--------	*Eco*RI & *Eco*RV	5'-GAATTC-3', 5'-GATATC-3'
Palmatine	448 bp	No Binding observed at 2 mM	----------	*Eco*R I & *Eco*RV	5'-GAATTC-3' 5'-GATATC-3'

[#]*Concentration of different anti-tumor drugs required to inhibit restriction endonucleases cleavage by 100%.*
[*]*Sequence selective binding of different anti-tumor drugs to the 448bp DNA fragment of pBCKS[+] plasmid.*

CONCLUSION

The result of our experiment with distamycin-A, adriamycin and daunomycin are similar to those the earlier works. The results obtained with the standards led to successful experimentation with mitoxantrone while the results obtained from restriction inhibition assay of mitoxantrone has shown that binding specificity for 5'-GC-3' is in accordance with that reported by Fox *et al* [1] and Panousis *et al* [3], who showed binding at CG, CA and TA (reading in the 5'-3' direction). Berberine and palmatine which were expected to show binding at 5'-ApT-3'or 5'-TpA-3' did not, even at millimolar concentration. Thus, RIA (restriction inhibition assay) is a sensitive and biologically relevant method to determine the binding concentration and sequence specificity of several anti-tumor drugs. Furthermore, this assay can be useful as a screening protocol for determining both sequence specificity and binding concentration of naturally-occurring and synthetic anti-tumor drug candidates. Additionally, this will be a useful tool to study the mechanism of action of several anti-tumor agents at the molecular level.

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