Differential effects of distamycin analogues on amplification of human gene sequences by polymerase-chain reaction

Marco PASSADORE,* Nicoletta BIANCHI,* Giordana FERIOTTO,* Carlo MISCHIATI,* Patrizio GIACOMINI,† Roberta PIVA* and Roberto GAMBARI*‡§

*Department of Biochemistry and Molecular Biology, Ferrara University, Ferrara, †Immunology Laboratory, Regina Elena Institute, Rome and ‡Biotechnology Centre, Ferrara University, Ferrara, Italy

In this report we analyse the effects of distamycin and five distamycin analogues on amplification by polymerase-chain reaction (PCR) of two gene sequences displaying a different A + T/G + C content. The first was a 5' region of the human oestrogen receptor (ER) gene, containing a (TA)₂₆ stretch; the second was a CG-rich sequence of the human Ha-*ras* oncogene. The results obtained unequivocally demonstrate that the addition of one pyrrole ring significantly improves the ability of distamycin derivatives to interfere with PCR-mediated amplification of the human ER genomic region carrying a (TA)₂₆ stretch. The distamycin analogues analysed differ in the number of pyrrole

rings and in the presence of an N-formyl, an N-formimidoyl or a retroamide group at position X₁. Among compounds carrying the same number of pyrrole rings, those carrying an N-formyl or an N-formimidoyl group retain a similar inhibitory activity. The retroamide analogues, on the contrary, are much less efficient in inhibiting PCR-mediated amplification of the 5' ER region. With respect to sequence selectivity both distamycin and distamycin analogues exhibit a sequence preference, since they do not inhibit PCR amplification of Ha-*ras* CG-rich gene regions, with the exception of a distamycin analogue carrying four pyrrole rings.

INTRODUCTION

DNA-binding drugs are therapeutical compounds of great interest in view of their potential sequence selectivity [1–5]. Among DNA-binding drugs, distamycin [6] and distamycin analogues are antibiotics that have been extensively studied and reported to exhibit strong anti-viral and anti-tumour activities [6,7]. Although their mechanism of action is still unknown, recent data suggest that they form a complex with a high degree of selectivity for AT-rich DNA sequences [6–8]. Because this binding induces conformational changes in DNA structure [9], it may interfere both with DNA–protein interactions (transcription initiation) as well as activity of RNA polymerase II (transcription elongation). DNA binding of distamycin is expected to be one of the molecular mechanism(s) of action of this drug, leading to its known overall cytotoxic effects [10–14].

Recently, evidence has been provided that distamycin inhibits the activity of proteins capable of associating with DNA, such as restriction enzymes, topoisomerase II, DNA ligase, RNA polymerase, DNA polymerases and transcription factors [10–14].

In this report we analyse the effects of distamycin and five distamycin analogues on amplification by PCR of two sequences displaying a different A + T/G + C content. The first was a 5' region of the human oestrogen receptor (ER) gene [15], containing a (TA)₂₆ stretch [16–18]; the second was a GC-rich sequence of the human Ha-*ras* oncogene [19].

The distamycin analogues analysed differ in the number of pyrrole rings and in the presence of an N-formyl, an N-formimidoyl or a retroamide group at position X₁.

We have exploited these model systems to identify distamycin analogues with improved DNA-binding abilities.

MATERIALS AND METHODS

DNA-binding drugs

The compounds analysed were distamycin (synonyms distamycin A, stallimycin) hydrochloride (MEN 10399), and two derivatives synthesized by addition of an N-formimidoyl (MEN 10397) or a retroamide (MEN 10400) at position X₁. In addition, we analysed the related analogues (MEN 10567, MEN 10559 and MEN 10706) carrying a fourth pyrrole ring. These compounds were obtained by total synthesis at the laboratories of Menarini Ricerche Sud, Pomezia (Rome, Italy). Daunomycin was from Sigma Chemical Co., St. Louis, MO, U.S.A.

Target DNA, oligonucleotide primers and PCR

The sequences and the locations of the primers used for PCR [20] are reported in Figure 1. *Taq* DNA polymerase (Perkin-Elmer, Cetus, U.S.A.) was added at a final concentration of 2.5 units/25 µl. The nucleotide sequence of a 3.2 kb genomic region located upstream of the ER gene sequence originally designated exon 1 was recently investigated and described in our laboratory [16,17]. This region contains sequences, presumably targets of distamycin, such as a (TA)₂₆ stretch located about 1.4 kb upstream of the exon 1 [18]. For the amplification of the 5' region of the human ER gene the target DNA was either the pBLCAT8ERCAT1 plasmid [15] or human genomic DNA.

The amplification primers for the codon 61 region of the Ha-*ras* oncogene were from the ras PointPrimers kit (Oncogene Sciences, Uniondale, NY, U.S.A.). For the amplification of the Ha-*ras* oncogene, the target DNA was from the Chinese hamster

Abbreviation used: ER, oestrogen receptor.

§To whom correspondence should be addressed at: Dipartimento di Biochimica e Biologia Molecolare, Università di Ferrara, Via L. Borsari n.46, 44100 Ferrara, Italy.

Figure 1 Location of PCR primers and DNA sequences of ER (a) and Ha-ras (c) PCR products and effects of distamycin on generation of F1/R1 ER PCR products (b) and of distamycin and daunomycin on generation of PCR-61 Ha-ras product (d)

(a) Exon -1 and exon 1 of the human ER gene are designated according to previously published results [16–18]. (b) PCRs were performed in the absence (lane a) or presence of 100 (lane b), 50 (lane c), 25 (lane d), 12 (lane e), or 6 (lane f) μ M distamycin. Target DNA was the pBLCAT8ERCAT1 plasmid DNA. (d) PCRs were performed in the absence (lanes a and i) or in the presence of 100 (lanes c and d), 50 (lanes b and e), 25 (lane f), 12 (lane g), 6 (lane h) μ M drugs concentrations. Target DNA was from FH06T1-1 cells [19]. Abbreviation: M, molecular-mass markers (*Hae*III-digested pBR322 plasmid DNA).

FH06T1-1 cell line [19], carrying multiple copies of the human Ha-ras oncogene in their genome.

The effects of DNA-binding drugs were analysed after incubating target DNA at room temperature, for 5 min, with increasing amounts of DNA-binding drugs, as reported in the text, followed by PCR. Amplified DNA was analysed by electrophoresis on 2.5% agarose, in TAE (0.04 M Tris/acetate, 0.001 M EDTA) containing 0.5 μ g/ml ethidium bromide. Conditions of Ha-ras PCR were: denaturation, 92 °C, 45 s; annealing, 62 °C, 45 s; elongation, 72 °C, 30 s. Conditions of ER PCR were: denaturation, 92 °C, 45 s; annealing, 55 °C, 1 min; elongation, 72 °C, 1 min.

Arrested sequencing and arrested PCR

Distamycin-mediated arrested sequencing was performed as follows: 50 ng of target DNA (2.5 kb *Bam*HI ER fragment) [16,17] was primed with 3 pmol of specific primer and sequencing was performed by the dideoxynucleotide chain termination method [21], with Sequenase Version 2.0 (US Biochemicals). ³²P-

labelled R1 ER primer (see Figure 1 for sequence and location) was used for sequencing reactions. The ³²P-labelled fragments were ethanol-precipitated and analysed on a sequencing gel.

Arrested PCR was performed by adding, after 20 cycles of F1/R1 PCR, ³²P-labelled R1 ER primer. After 10 additional PCR cycles performed in the presence or in the absence of DNA-binding drugs, the ³²P-labelled fragments were ethanol-precipitated and analysed on a sequencing gel. Control sequencing reactions were performed by the dideoxynucleotide chain termination method: PCR reactions were loaded in a 0.8% low-melting-point agarose gel; the specific DNA band was cut, melted at 50 °C and a volume corresponding approximately to 0.05 pmol was primed with 3 pmol of R1 specific primer and sequenced.

RESULTS

Effects of distamycin on PCR-mediated amplification of different human gene regions

Figure 1 shows the effects of distamycin on the PCR-mediated

(a) Sequencing reactions were performed in the absence (0) or in the presence of 1.25, 2.5 and 5 μM distamycin. The DNA fragment to be sequenced was the 2.5 kb *Bam*HI ER fragment [16,17], primed with 3 pmol of specific ^{32}P -labelled R1 ER primer. Sequencing reactions were performed using the dideoxynucleotide chain termination method [21] with Sequenase Version 2.0 (US Biochemicals). The (TA)₂₆ stretch is indicated. (b) 1 μg of human genomic DNA was incubated in PCR conditions with the F1 and R1 primers in the absence of distamycin. After 20 cycles of F1/R1 PCR, ^{32}P -labelled R1 ER primer was added and the reaction was carried on for 10 additional cycles in the presence of 0.5, 2.5, 5 and 20 μM distamycin. The ^{32}P -labelled products were analysed on a sequencing gel (b) and compared with a sequencing reaction performed by the dideoxynucleotide chain termination method (a). Arrows indicate distamycin-dependent arrest sites corresponding to the (TA)_n stretch. The DNA sequences corresponding to arrest sites are shown in the left-hand side of the panel.

The results obtained are shown in Figures 1(b) and 1(d) and clearly demonstrate that distamycin inhibits PCR-mediated amplification of the 5' portion of the human ER gene, being

Inhibition of the PCR-mediated amplification of the 5' region of the human ER gene by distamycin was reproducibly obtained in seven independent experiments employing both plasmid DNA and human genomic DNA as target sequences (data not shown).

Identification by arrested sequencing and arrested PCR of the DNA sequences preferentially recognized by distamycin

In order to further confirm the sequence selectivity of binding of distamycin to the 5' portion of the ER gene, the experiment shown in Figure 2(a) has been performed. Sequencing of the 2.5 kb *Bam*HI ER fragment was performed by the dideoxynucleotide chain termination method [21] in the absence or in the presence of 1.25–5 μ M distamycin. 32 P-labelled R1 ER primer was used in these sequencing reactions.

The results of this experiment suggest that distamycin blocks the sequencing reaction at the level of the (TA)₂₆ stretch. The labelled sequencing fragments corresponding to the (TA)₂₆ stretch, as well as fragments of higher molecular mass, are indeed under-represented in the sequencing reaction carried on in the presence of distamycin. These results suggest that distamycin selectively binds to the (TA)₂₆ stretch of the 5' region of the human ER gene. Accordingly, no alteration by 5 μ M distamycin was observed when sequencing was performed on ER genomic control regions lacking the (TA)₂₆ stretch (data not shown). By contrast, no blockage of sequencing reactions was obtained when 5 μ M daunomycin is added (not shown).

Since distamycin binds to duplex DNA, one likely explanation of this experiment is that the drug binds and stabilizes a secondary hairpin/loop structure occurring at the level of the (TA)₂₆ tract, thereby preventing the polymerase from functioning. Therefore, the sequence selectivity of distamycin was further investigated by arrested PCR. In this experiment (shown in Figure 2b) F1/R1 PCR was performed. After 20 cycles of F1/R1 PCR, 32 P-labelled R1 ER primer was added and the reaction was carried on for 10 additional cycles in the presence or absence of distamycin. The 32 P-labelled products were analysed on a sequencing gel and compared with a sequencing reaction performed by the dideoxynucleotide chain termination method. The results obtained clearly indicate that at 2.5 μ M distamycin concentration at least five abortive sites are detectable, three of which are located within the (TA)₂₆ stretch. Interestingly, the other two sites detected are close to sequences 5'-TGTATG-3' and 5'-GTTTAAC-3' (Figure 2b). The data clearly indicate that the inhibition of PCR amplification by distamycin occurs at specific AT-rich sites. In addition, these data suggest that efficient annealing between PCR primers and denatured DNA template occurs in the presence of distamycin, and that distamycin does not prevent melting of the target DNA. This is in agreement with data reviewed elsewhere on the interactions between these DNA-binding drugs and DNA [22,23]. Control experiments demonstrate that, fully in agreement with data shown in Figure 2, daunomycin does not display any PCR abortive sites even if added at a concentration of 20 μ M (data not shown).

Effects of distamycin analogues on PCR-mediated amplification of the ER and Ha-ras gene regions

The chemical structures of the distamycin analogues studied are depicted in Figure 3. Figure 4(a) shows representative data on the efficiency of PCR-mediated amplification of the ER 5' region in the presence of distamycin analogues. Figure 4(b) shows Ha-ras PCRs performed in the presence of concentrations of distamycin analogues demonstrated to be active in suppressing or inhibiting amplification of the ER 5' region. Quantitative analyses are reported in Figure 5. Differential inhibition of F1/R1 ER PCR by distamycin and distamycin analogues is also obtained, consistently with data shown in Figure 4(a), when these DNA-binding drugs are exposed to 1, 2, 3, 5, 10 and 20 PCR cycles in the absence of DNA template, primers and *Taq* DNA polymerase, followed by 30 cycles of PCR amplification

Figure 3 Chemical structures of distamycin (10399) and the distamycin analogues used in the present study

These compounds were obtained by total synthesis at the laboratories of Menarini Ricerche Sud, Pomezia (Rome, Italy).

(data not shown). These data suggest that the effects presented in Figure 4(a) are not due to differential stability of the compounds under the experimental conditions employed.

Figure 4 Effects of distamycin and distamycin analogues on PCR amplifications of F1/R1 ER (a) and Ha-ras codon 61 (b) regions

Target DNA was human genomic DNA (ER PCR amplifications) and DNA from FH06T1-1 cells (Ha-ras PCR amplifications). Arrows indicate specific PCR products. PCRs were performed in the absence (lane h) or in the presence of 125 (lane a), 100 (lane b), 50 (lane c), 25 (lane d), 12 (lane e), 6 (lane f) and 3 (lane g) μM drug concentrations. Hybridization of the agarose gel to an ER-specific ^{32}P -labelled probe demonstrates that the lower-molecular-mass bands which are evident in (a) are ER-specific, suggesting that, in agreement with results from other laboratories [13], they are generated by DNA bending. Abbreviation: M, molecular-mass markers (*Hae*III-digested pBR322 plasmid DNA).

Taken together, the results obtained suggest that, among compounds carrying three (10399, 10397 and 10400) pyrrole rings, 10399 is slightly more effective than the relative N-formimidoyl analogue (10397), but much more effective than the retroamide analogue (10400) in inhibiting PCR-mediated amplification of the 5' ER region. Accordingly, among compounds carrying four (10567, 10559 and 10706) pyrrole rings, 10567 is slightly more effective than the relative N-formimidoyl analogue (10559), but much more effective than the retroamide analogue (10706) in inhibiting F1/R1 ER PCR.

When distamycin analogues carrying three pyrrole rings are compared with those carrying four pyrrole rings it appears that, with no exception, the addition of a fourth pyrrole ring confers to the distamycin analogues higher inhibitory activity.

The effects of the same compounds on PCR-mediated amplification of the codon 61 region of the human Ha-ras oncogene gives information on their sequence selectivity. The results obtained are shown in Figures 4(b) and 5(b) and indicate that distamycin and distamycin analogues are fairly sequence-specific. In fact, they do not inhibit, the PCR-mediated amplification of Ha-ras oncogene sequences, even when added at a final concentration of 100 μM . Only compound 10567 was found to inhibit Ha-ras PCR.

Control experiments indicate that 100 μM daunomycin and

the GC-specific mithramycin and chromomycin [24] do not inhibit ER PCR, being on the contrary effective in suppressing PCR-mediated amplification of Ha-ras genomic sequences (data not shown).

DISCUSSION

In this study we have introduced chemical modifications in the original distamycin molecule to alter the number of pyrrole rings, charge and planarity of these molecules, with the aim of comparatively assessing the ability of five novel distamycin derivatives to interfere in a sequence-specific manner with PCR-mediated amplification of human genomic regions.

In this paper we compared the *in vitro* effects of distamycin analogues, employing as a model system the human ER gene [15] and the Harvey-ras oncogene [19], in order to obtain PCR products differing significantly for the A+T/G+C frequency ratio. Distamycin is indeed known to preferentially bind to AT-rich gene regions [7,8,10–12]. This was confirmed by experiments employing arrested sequencing and arrested PCR (Figure 2).

The results obtained unequivocally demonstrate that the addition of one pyrrole ring significantly improves the ability of distamycin derivatives to interfere with PCR-mediated

Figure 5 Quantitative analysis of the effects of distamycin and distamycin analogues on PCR amplifications of F1/R1 ER (a) and Ha-ras codon 61 (b) regions

Data reported were derived from the experiments shown in Figure 4. ●, MEN 10399; ▲, MEN 10397; ■, MEN 10400; ○, MEN 10567; △, MEN 10559; □, MEN 10706.

amplification of the human ER genomic regions carrying a (TA)₂₆ stretch.

Among compounds carrying the same number of pyrrole rings, those carrying an N-formyl or an N-formimidoyl group retain a similar inhibitory activity. The retroamide analogues, on the contrary, are much less efficient in inhibiting PCR-mediated amplification of the 5' ER region.

When we consider the chemical structure of the most active compounds (see Figure 3), we can conclude that the N-formimidoyl group appears to confer, under our experimental conditions, better sequence selectivity. Compound 10559 inhibits the ER PCR with an efficiency similar to that of 10567, but does not affect Ha-ras amplification, while compound 10567 fully suppresses the production of Ha-ras PCR products when added at 100 μM concentrations.

We therefore propose this approach as a useful system to identify DNA-binding drugs displaying sequence selectivity even at high concentrations.

These results are in agreement with DNase I footprinting experiments performed on HIV-1 LTR (G. Feriotto, C. Mischiati and R. Gambari, unpublished work), suggesting that both distamycin and the distamycin analogues here studied bind to the TATAA TFIID element, displaying efficiencies similar to those found in the present paper using the PCR approach. In addition, band-shift experiments were performed with synthetic double-stranded oligonucleotides containing either (a) the Sp1 (GGG-GCGGGG) or (b) the octamer (ATGCAAAT) binding motifs and (c) both crude nuclear extracts and recombinant binding proteins. The data obtained demonstrate that (a) most of the distamycin analogues do not interfere with the binding between Sp1 and the target DNA element (G. Feriotto, C. Mischiati and R. Gambari, unpublished work) and (b) the interaction between octamer-binding proteins and synthetic oligonucleotides is differentially inhibited by distamycin and distamycin analogues [25], in agreement with the data reported in Figures 4 and 5.

Taken together, these data suggest that the distamycin analogues studied exhibit differential effects which are reproducibly observed when different molecular approaches (band-shift, DNase I footprinting and PCR) are employed.

Concerning the general issue of the choice of convenient approaches to study the effects of DNA-binding drugs our results therefore suggest that PCR technology could be applied to compare *in vitro* effects of DNA-binding drugs with similar structure.

A second conclusion that can be drawn from the experiments here reported is that the distamycin analogue 10559 retains the highest activity and the better sequence selectivity in inhibiting PCR.

The experimental approach here described has, to our mind, an additional important advantage over other molecular analyses of DNA-drug interactions. PCR is a very flexible technique and, in principle, could be applied in pharmacological studies of DNA-binding drugs in *ex vivo* experiments employing cell culture systems. In this respect, PCR can be performed directly on permeabilized cells treated with a given DNA-binding drug in order to determine whether the compound reaches intracellular concentrations, allowing efficient binding to selected genomic regions. In the presence of pharmacologically damaged DNA, PCR could also bring information on the interactions of DNA-binding drugs with specific genomic regions in intact cells or in selected tissues.

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