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In vitro and in vivo binding of a CC-1065 analogue to human gene sequences: a polymerase-chain reaction study

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Abstract

In this paper we analyse the in vitro sequence selectivity of the CC-1065 analogue 2-[[5-[(1*H*-indol-2-ylcarbonyl)-1*H*-indol-2-yl]carbonyl]-7-methyl-1,2,8,8*a*-tetrahydrocyclopropa[*c*]-pyrrolo-[3,2-*e*]-indol-4-one (U-71184) employing the polymerase-chain reaction (PCR). In addition, we determined whether alteration of PCR by U-71184 is detected when DNA is isolated from cells cultured in the presence of this drug. As molecular model systems we employed the human estrogen receptor gene, the Ha-ras oncogene and the chromosome X-linked, (CGG)-rich fragile X mental retardation-1 gene. The first conclusion that can be drawn from the experiments reported in our paper is that U-71184 inhibits PCR in a sequence-dependent manner. A second conclusion of our experiments is that PCR performed on DNA from U-71184-treated cells is inhibited when the primers amplifying the estrogen receptor gene region are used. This approach might bring important information on both in vivo uptake of the drug by target cells and binding to DNA.

Keywords: CC-1065 analog; Gene sequence, human; PCR (polymerase-chain reaction)

1. Introduction

Recent published observations from a large number of molecular biology laboratories demonstrate that natural antibiotics, such as the DNA-binding drug CC-1065 (Hurley et al., 1988; Warpehoski et al., 1992), interfere with the activity of a large variety of DNA-binding proteins, including transcription factors (Broggini et al., 1989; Gambari et al., 1991; Dorn et al., 1992; Gambari and Nastruzzi, 1994; Feriotto et al., 1994a; Welch et al., 1994). This could have important practical applications in the experimental therapy of many human pathologies, including neoplastic as well as infectious diseases (Gambari and Nastruzzi, 1994). It is well established, indeed, that regulation of gene expression is operated, at the transcriptional level, by complex interactions between proteins (transcription factors) and promoters containing specific target elements (Berg and Von Hippel, 1988; Mitchell and Tjian, 1989; Lewin, 1991; Faisst and Meyer, 1992; Gaynor, 1992). From these considerations, it is hypothesized that pharmacologically mediated modulation of DNA/nuclear protein complexes formation could represent a promising approach to control expression of selected genes of eukaryotic cells (Dervan, 1986; Lown, 1988; Laughton et al., 1990; Gambari and Nastruzzi, 1994; Feriotto et al., 1995). To test this hypothesis, a large number of investigations on sequence selectivity of DNA-binding drugs were recently published employing footprinting and gel retardation studies (Dervan, 1986; Portugal and Waring, 1987; Lown, 1988; Broggini et al., 1989; White and Phillips, 1989; Churchill et al., 1990; Fox et al., 1990; Laughton et al., 1990; Gambari et al., 1991; Gambari and Nastruzzi, 1994; Dorn et al., 1992; Feriotto et al., 1994a,b; Welch et al., 1994). Few information, on the contrary, is available on the in vivo interaction of DNA-binding drugs with specific tissues and selected genes. This information is of great interest, as sequence-selective binding of DNA-binding drugs might affect the activity of RNA polymerase only in genes that contain short DNA sequences preferentially recognized by the compounds (Gambari and Nastruzzi, 1994).

One of the DNA-binding drugs exhibiting sequence selectivity is CC-1065, a potent antitumor antibiotic pro-

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Gene	PCR product	bp	(A+T)/(G+C)
ER gene (5' genomic region)	GACGCATGATATACTTCACCTATTTTTTGTCTCCTTAATA TGTATATATATATATATATATATATATATATATATATATAT ATATATATATATATATATACACATATATGTGTGTGTGTAT GTGCGTGTGCATGTTTAACTTTTAATTCAGTTAAAAACT TTTTTCTATTTGTTTTTCATCTGGATATTTGATTCTGC	182	3.46
Ha-ras-1 oncogene	AGACGTGCCTGTTGGACATCCTGGATACCGCCGGCCA GGAGGAGTACAGCGCATGCGGGACCAGTACATGCG	72	0.6
FMR-1 gene	GACGGAGGCGCCCGTGCCAGGGGGCGTGCGGCAGCGCGGCG GCGGCGGCGGCGGCGGCGGCGGCGGAGGCGGCGGCGGCGGCG GCGGCGGCGGCGGAGGCGGCGGCGGCGGCGGCGGCGGCGGCG GCTGGGCCTCGAGCGCCCGCAGCCCACCTCTCGGGGGCGGGC TCCCGGCGCTAGCAGGGCTGAAGAGAAGATGGAGGA	206	0.46

Fig. 1. Nucleotide sequences of PCR primers (underlined) and PCR products; in the right column the (A + T)/(G + C) ratios are also shown.

duced by *Streptomyces zelensis*, able to bind to DNA through N3 of adenine (Hurley et al., 1988; Aristoff, 1993). This minor groove binder constitutes three repeating pyrrolindole subunits, one of which contains a reactive cyclopropyl function. CC-1065 is known to inhibit transcription factor IID binding to target DNA sequences (Welch et al., 1994), similarly to other minor groove binders, including distamycin and berenil (Feriotto et al., 1994a; Welch et al., 1994).

In this paper we analyze the in vitro effects of the CC-1065 analogue 2-[[5-[(1*H*-indol-2-ylcarbonyl)-1*H*-indol-2-yl]carbonyl]-7-methyl-1,2,8,8*a*-tetrahydrocyclopropa-[*c*]-pyrrolo-[3,2-*e*]-indol-4-one (U-71184) (Warpehoski, 1986) employing a recently described polymerase-chain reaction (PCR) approach (Passadore et al., 1994, 1995). In addition, we determined whether alteration of PCR by U-71184 is detected when DNA is isolated from cells cultured in the presence of this drug. U-71184 appears to be a very interesting antitumor compound, being 10-fold more potent than CC-1065 in the inhibition of L210 leukemia cell growth (Hurley et al., 1988). Studies on in vivo activity of U-71184 against P388 leukemia demonstrate that this compound produces multiple long-term survivors. By contrast, CC-1065 causes in the same model system a 67% decrease in life span (Hurley et al., 1988; Warpehoski et al., 1992; Aristoff, 1993).

In the experiments reported in the present paper we employed as molecular model systems the human estrogen receptor gene (Green et al., 1986; Piva et al., 1992), the Ha-ras oncogene (Santos et al., 1982) and the chromosome X-linked, (CGG)-rich, fragile X mental retardation-1 gene (FMR-1) (Erster et al., 1992) for a number of endpoints. We have published the nucleotide sequence of a 3.2 kb genomic region located upstream of the estrogen receptor sequence originally designated exon 1 (Piva et al., 1992; Piva and Del Senno, 1993) and demonstrated that this region contains A + T-rich sequences recognized by distamycin (Del Senno et al., 1992) and, presumably, by U-71184 (Hurley et al., 1988; Aristoff, 1993). Both Ha-ras and FMR-1 genomic sequences, on the other hand, are G + C rich and therefore could interact with low efficiency with U-71184.

2. Materials and methods

2.1. Drugs and enzymes

U-71184 (lot No. 18772-PDJ-104) was obtained from the Upjohn Company (Kalamazoo, MI, USA). Distamycin and chromomycin were from Sigma (st. Louis, MO, USA). Stock solutions of U-71184 (45.2 mM), distamycin (9.6

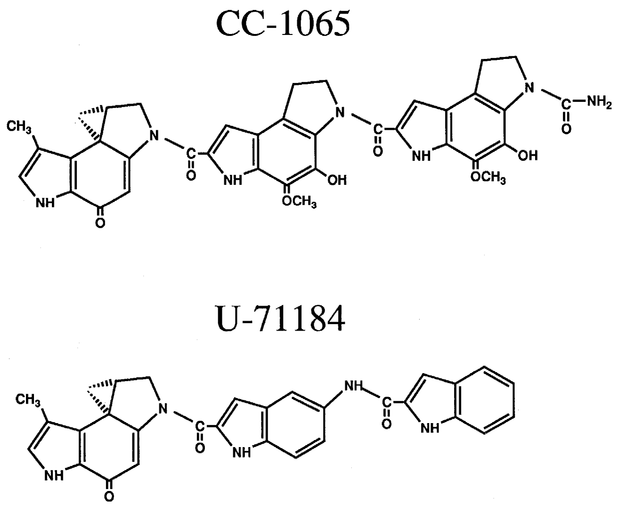

Fig. 2. Chemical structure of CC-1065 and U-71184.

mM) and chromomycin (0.84 mM) were stored at −20°C in the dark and diluted immediately before use.

2.2. Cell culture conditions

The MCF7 human breast cancer cell line (Piva et al., 1993) was cultured in MEM α-medium supplemented with 10% fetal calf serum (Gibco, Gaithersburg, MD, USA), in 5% CO_2 humidified atmosphere.

2.3. Target DNA, oligonucleotide primers and PCR

The sequences of the primers used for PCR (Saiki et al., 1988) are reported in Fig. 1. *Taq* DNA polymerase (Per-

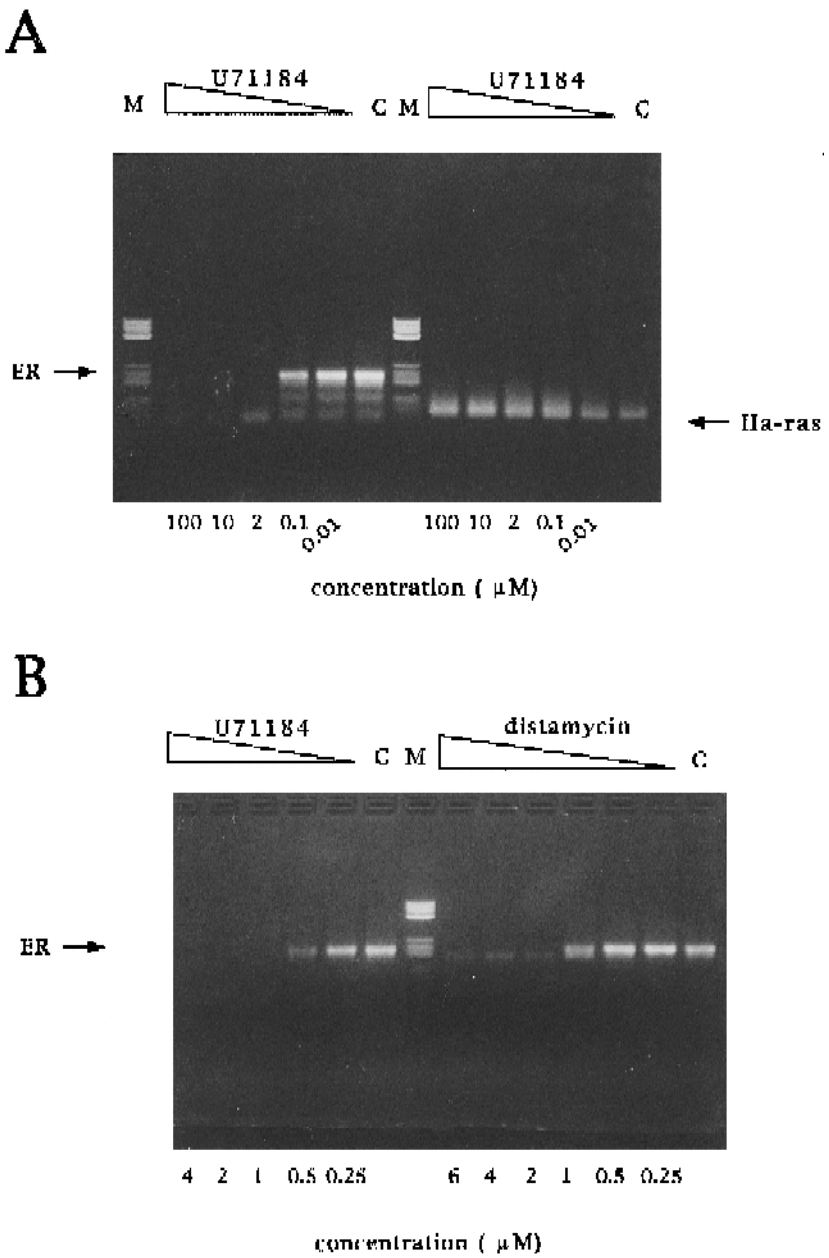

Fig. 3. (A) Effects of U-71184 on PCR-mediated amplification of estrogen receptor (left) and Ha-ras (right) sequences. (B) Effects of U-71184 and distamycin on PCR-mediated amplification of estrogen receptor sequences. PCRs were performed in the absence (C) or in the presence of the indicated (0.01–100 μM) concentrations of U-71184. Target DNA was 50 ng of PCR products obtained by amplification of plasmid pBLCAT8ERCAT1 (containing estrogen receptor sequences) (Green et al., 1986) and 0.5 μg of FH06T1-1 cell DNA (containing multiple copies of the human Ha-ras oncogene) (Spandidos and Wilkie, 1984). M = molecular weight marker (*Hae*III-restricted pBR322 plasmid DNA). Estrogen receptor and Ha-ras PCR products are indicated by arrows.

kin-Elmer Cetus, Norwalk, CT, USA) was added at 2.5 U/25 μl final concentration. The nucleotide sequence of a 3.2 kb genomic region located upstream of the estrogen receptor sequence originally designated exon 1 was investigated and described in our laboratory (Piva et al., 1992; Piva and Del Senno, 1993). This region contains sequences, presumably targets of U-71184 (Del Senno et al., 1992; Piva et al., 1993). For the amplification of the 5′ region of the human estrogen receptor gene the target DNA was either the pBLCAT8ERCAT1 plasmid (Green et al., 1986) or human genomic DNA.

The amplification primers for codon 61 regions of the Ha-ras oncogene were from the ras PointPrimers kit (Oncogene Sciences, Uniondale, NY, USA). For the amplification of the Ha-ras oncogene, the target DNA was from the Chinese hamster FH06T1-1 cell line (Spandidos and Wilkie, 1984), carrying multiple copies of the human Ha-ras oncogene in their genome.

After PCR-mediated amplification of the (CGG)-rich region of the X-linked FMR-1 gene the specificity of the PCR products was demonstrated by Southern blotting and hybridization with the internal ^{32}P-labeled probe $(CGG)_5$ (Erster et al., 1992).

The effects of DNA-binding drugs were analyzed after incubating target DNA at room temperature, for 5 min, with increasing amounts of the compounds, as reported in the text, followed by PCR. Amplified DNA was analyzed by electrophoresis on 2.5% agarose, in TAE (0.04 M Tris-acetate, 0.001 M EDTA) and 0.5 μg/ml ethidium bromide. Conditions of Ha-ras PCR were: denaturation, 92°C, 45 s; annealing, 62°C, 45 s; elongation, 72°C, 30 s (32 cycles); conditions of estrogen receptor PCR were: denaturation, 92°C, 45 s; annealing, 55°C, 1 min; elongation, 72°C, 1 min (32 cycles). Conditions of FMR-1 PCR were: denaturation, 94°C, 1 min; annealing, 65°C, 1 min; elongation, 72°C, 1.5 min (32 cycles).

3. Results

3.1. Effects of U-71184 on amplification of the ER 5′ region by PCR

In Fig. 1 the PCR primers used to amplify the estrogen receptor, the Ha-ras and the FMR-1 genomic sequences are indicated, together with the nucleotide sequence of the PCR products obtained. As it is evident by comparing the nucleotide sequences reported in Fig. 1, only the estrogen receptor PCR product contains A + T-rich sequences presumably recognized by U-71184. Accordingly, Fig. 1 shows that the A + T/G + C ratios are sharply different in the estrogen receptor, Ha-ras, and FMR-1 PCR products. The A + T/G + C ratio of the estrogen receptor product is 3.46, while the A + T/G + C ratios of Ha-ras-1 and FMR-1 PCR products are 0.6 and 0.46, respectively. In Fig. 2 the chemical structures of CC-1065 and its analogue U-71184 are shown.

Fig. 3A (left panel) shows that U-71184 inhibits the PCR when the estrogen receptor specific primers are used. This experiment was performed using an estrogen receptor PCR product as target DNA (50 ng/PCR). By contrast, the right panel of Fig. 3A shows that no inhibition by U-71184 was obtained when Ha-ras PCR was performed. Fig. 3B shows a comparison of the activity of U-71184 and distamycin on PCR-mediated amplification of estrogen receptor sequences. The results obtained show that both U-71184 and distamycin inhibit estrogen receptor PCR, while at different levels, with U-71184 being more active than distamycin. Fifty percent inhibition of the generation of estrogen receptor PCR products is achieved with 0.5 μM distamycin and 0.25 μM U-71184, respectively. No inhibition of estrogen receptor PCR was obtained even in the presence of 50 μM mithramycin and chromomycin (data not shown and Passadore et al., 1994). Interestingly,

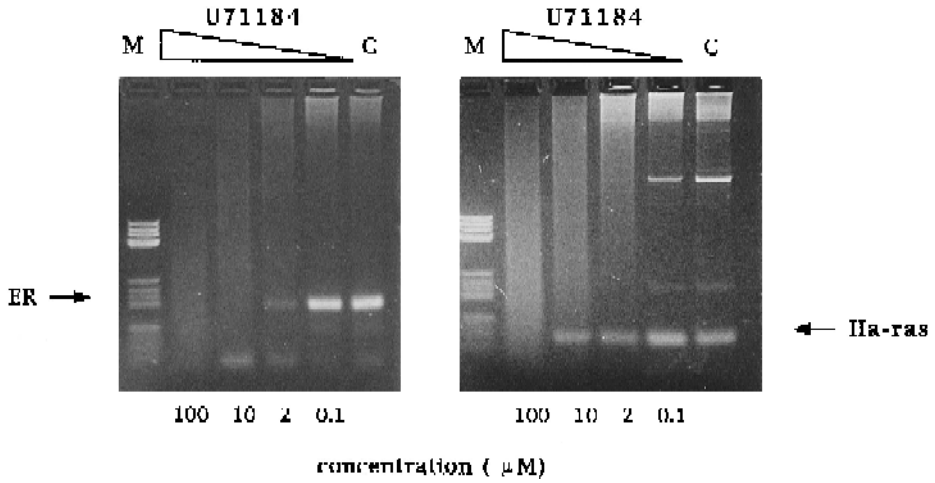

Fig. 4. Effects of U-71184 on PCR-mediated amplification of estrogen receptor (left panel) and Ha-ras gene (right panel) sequences performed on 0.5 μg of target human genomic DNA. PCRs were performed in the absence (C) or in the presence of the indicated (0.1–100 μM) concentrations of U-71184. M = molecular weight marker (*Hae*III-restricted pBR322 plasmid DNA).

mithramycin and chromomycin are known to bind to GC-rich sequences (Van Dyke and Dervan, 1983; Ray et al., 1989; Snyder et al., 1991). Accordingly, both mithramycin and chromomycin inhibit Ha-ras PCR (Passadore et al., 1994 and data not shown).

3.2. Effects of U-71184 on PCR performed with human genomic DNA

Fig. 4 shows that inhibition of estrogen receptor PCR by U-71184 occurs also when human genomic DNA is employed as target DNA. In agreement with a sequence-selective effect of U-71184 and with the results presented in Fig. 3, U-71184 at 2 μM concentration is effective in inhibiting estrogen receptor PCR (Fig. 4, left side of the panel). U-71184 does not affect PCR-mediated amplification of genomic regions containing GC-rich sequences, such as the Ha-ras oncogene (Fig. 4, right side of the panel). The lack of PCR-mediated amplification of Ha-ras gene sequences when genomic DNA is treated with 100 μM U-71184 is due to a degradation of genomic DNA, as clearly evidenced by looking at the ethidium bromide staining of the agarose gel (Fig. 4, right side of the panel).

3.3. PCR on DNA from U-71184-treated MCF7 cells

An obvious application of the PCR approach to study drug/DNA interactions is related to in vivo studies that

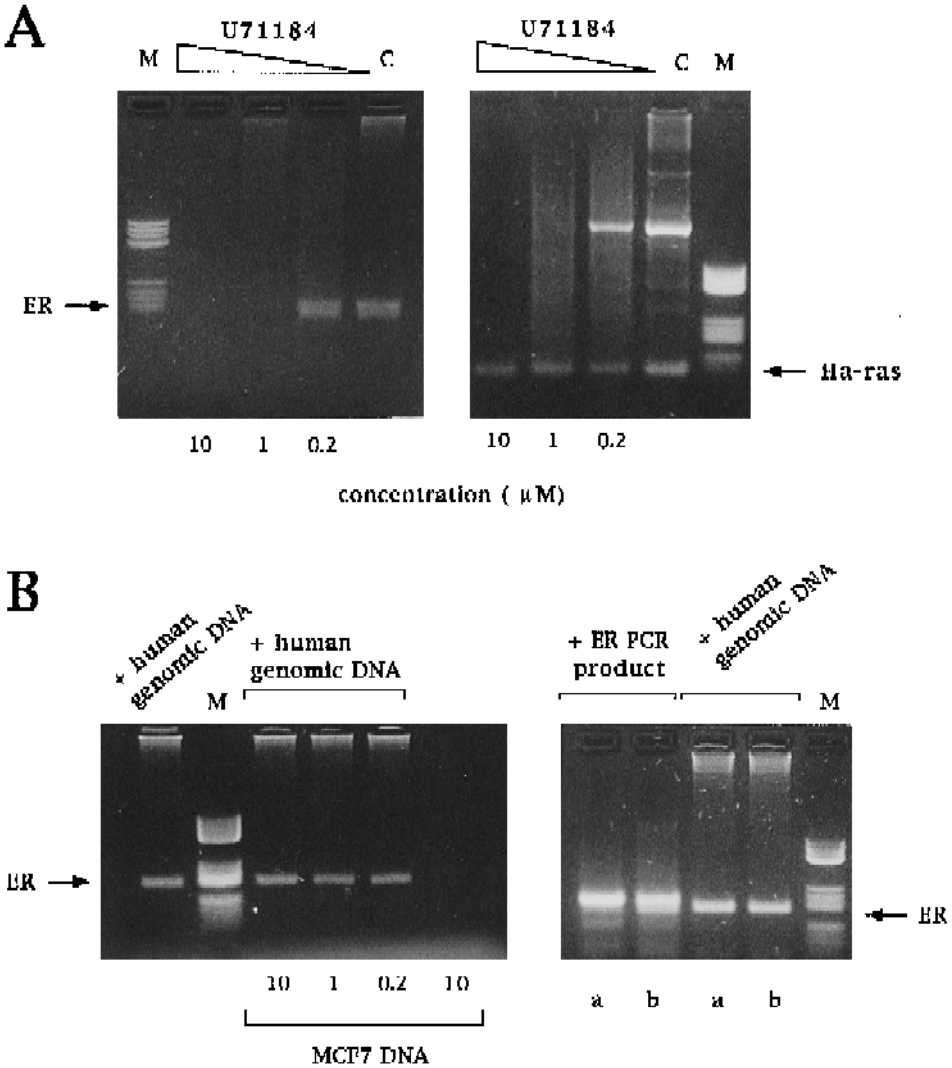

Fig. 5. (A) In vivo effects of U-71184. MCF7 breast cancer cells were cultured for 24 h in the absence (C) or in the presence of the indicated concentrations (0.2, 1 and 10 μM) of U-71184. Cells were collected, lysed as described in Section 2, DNA was isolated and 0.5 μg was added to PCR mixtures. The estrogen receptor specific and Ha-ras specific PCR products are indicated by arrows. (B) Effects of the addition 0.5 μg of human genomic DNA or 50 ng of estrogen receptor PCR products (as indicated) to PCR mixtures performed with 0.5 μg of genomic DNA from MCF7 cells treated with the indicated concentration of U-71184. No DNA from U-71184 treated MCF7 cells was present in the reaction mixture analyzed in the first lane of the left panel and in the 'a' lanes of the right panel; in the 'b' lanes DNA from MCF7 cells treated with 10 μM U-71184 was added. M = molecular weight marker (*Hae*III-restricted pBR322 plasmid DNA).

can be carried out to determine, for instance, whether U-71184 binds to the 5′ upstream region of the estrogen receptor gene of intact cells in a dose-dependent manner. This is a very important point that differentiates this approach from footprinting and band-shift techniques (Passadore et al., 1994).

The experiment was carried out as follows: (a) human breast cancer MCF7 cells were cultured for 1 day in the presence of increasing amounts (0.5, 1 and 10 μM) of U-71184; (b) cells were pelleted, washed three times with PBS 1 × (phosphate-buffered saline) and DNA was phenol extracted and isolated; (c) PCR was performed on DNA from the same number of cells.

Fig. 5A (left panel) shows that specific estrogen receptor PCR products are not detected when genomic DNA from MCF7 cells treated with 1–10 μM of U-71184 is used. To confirm our hypothesis, PCR was performed using the Ha-ras specific PCR primers. The results are shown in the right panel of Fig. 5A and demonstrate that PCR products are, in this case, obtained even when the target DNA is isolated from MCF7 cells treated with 10 μM U-71184.

Control experiments demonstrated that the addition of untreated human genomic DNA or estrogen receptor PCR products to the PCR mixture containing DNA from U-71184-treated MCF7 cells leads to a PCR-mediated generation of estrogen receptor gene products (Fig. 5B), suggesting that the lack of amplification of estrogen receptor genomic sequences observed from the experiment shown in Fig. 5A is due to a change of genomic DNA isolated from U-71184-treated MCF7 cells.

Taken together, these results are compatible with in vivo binding of U-71184 to the 5′ region of the human estrogen receptor gene.

3.4. Effects of U-71184 on PCR-mediated amplification of FMR-1 CG-rich gene sequences

Fig. 6 shows that inhibition of FMR-1 PCR by U-71184 is not detected when human genomic DNA is used (A).

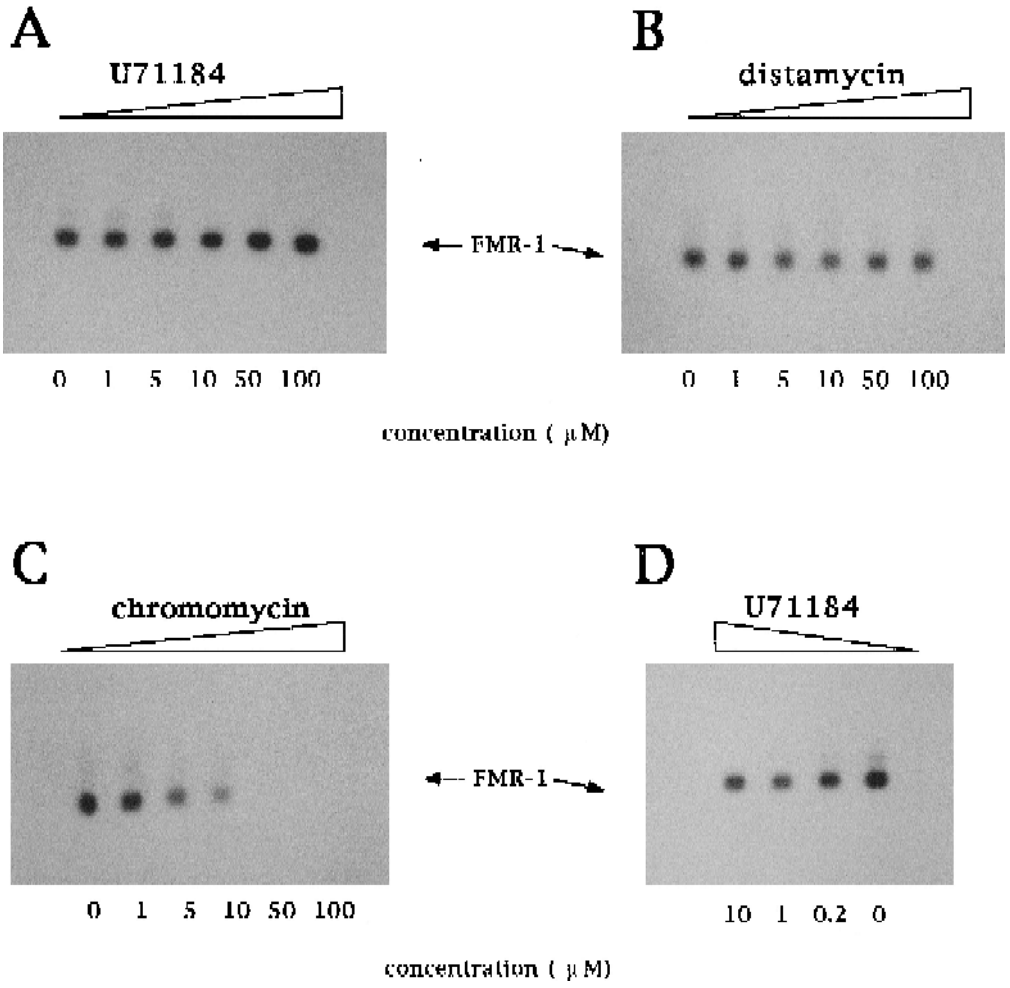

Fig. 6. Effects of U-71184 (A, D), distamycin (B) and chromomycin (C) on PCR-mediated amplification of FMR-1 gene sequences. Target DNA was 0.5 μg of human genomic DNA (A–C) or DNA isolated from MCF7 cells treated with the indicated concentrations (0.2, 1 and 10 μM) of U-71184. After 24-h treatment, MCF7 cells were collected, lysed as described in Section 2, DNA was isolated and added to PCR mixtures. Following PCR-mediated amplification, PCR products were Southern blotted and hybridized to an internal $(CGG)_5$ ^{32}P-labeled probe, as described in Section 2. The FMR-1 specific hybridization signals are arrowed.

Accordingly, also distamycin was found to be non-effective (B), while, as expected, chromomycin inhibits FMR-1 PCR amplification, in agreement with a selectivity of this compound for CG-rich genomic sequences. In this experiment, PCR products were Southern blotted and hybridized with a ^{32}P-labeled $(CGG)_5$ internal probe. The results shown in Fig. 6D demonstrate that FMR-1 PCR products are obtained even when the target DNA was isolated from MCF7 cells treated with 10 μM U-71184.

4. Discussion

A number of recent reports suggest that some antiviral and antitumor drugs could act through sequence-selective interactions with DNA, leading to alteration of DNA/nuclear protein complexes (reviewed in Dervan, 1986; Gambari and Nastruzzi, 1994). For instance, we (Gambari et al., 1991; Feriotto et al., 1994a,b, 1995; Gambari and Nastruzzi, 1994) and others (Broggini et al., 1989; Dorn et al., 1992; Chiang et al., 1994; Welch et al., 1994) have recently reported that distamycin is a strong inhibitor of the interaction between a variety of DNA-binding proteins (such as the transcription factor IID, the human TATA-box binding protein, the Epstein-Barr virus nuclear antigen 1, the Octamer binding protein I, the nuclear factor erythroid I, the nuclear factor Y, *Antennapedia* and *fushi-tarazu* homeodomains) and target DNA sequences. By contrast, distamycin does not inhibit the interactions between target DNA sequences and jun/fos, the transcription specific factor 1, EGR1 (early growth response gene-1) and WT1 (Wilms tumor suppressor gene-1) (Welch et al., 1994; Feriotto et al., 1994a).

The main issue of the present communication was to verify whether PCR technology could be applied to compare in vitro to in vivo effects of DNA alkylating drugs, such as CC-1065 analogues. The interest on CC-1065, from the pharmacological point of view, is that this compound is, up to now, one of the most potent inhibitors of in vitro tumor cell growth, being its activity at least 100 times higher than that exhibited by doxorubicin (Hurley et al., 1988; Aristoff, 1993). This compound appears to retain sequence selectivity, causing DNA alkylation after covalent binding to N3 of adenine in the minor groove of DNA. Analysis of the DNA sequences flanking the covalent binding site of CC-1065 suggests that this drug selectively interacts with the consensus sequence N-N-A/T-A/T-A-N-N (underlined is the bound adenine) (Hurley et al., 1988, 1990).

However, a drawback in the use of CC-1065 as antitumor drug in vivo is its high hepatotoxicity, leading to delayed death in mice treated with therapeutic doses (Warpehoski et al., 1992; Aristoff, 1993). This finding, on the one hand, precluded the clinical use of CC-1065, but on the other hand stimulated investigations to prepare CC-1065 analogues retaining similar or even higher activity without the side effects of CC-1065.

With respect to this issue, of great interest is the development of rapid protocols for the identification of DNA-binding drugs exhibiting sequence selectivity in DNA recognition. Among the molecular biology approaches so far proposed, our group has recently demonstrated that PCR could be considered as a fast, non-radioactive method to determine sequence selectivity in drug/DNA recognition (Passadore et al., 1994). This protocol could provide information on the activity of DNA-binding drugs both in vitro and in vivo, expecially if drug/DNA adducts are expected to be generated. In our experiments we used the CC-1065 analogue U-71184. This compound was selected because its efficacy against a broad spectrum of mouse tumor models, as well as human tumor xenograft (Warpehoski et al., 1992; Aristoff, 1993; Mohamadi et al., 1994). It should be pointed out that no significant problem was identified in studies performed to search for any evidence of delayed or irreversible toxicity at therapeutic doses (Aristoff, 1993).

The first conclusion that can be drawn from the experiments reported in our paper is that U-71184 inhibits PCR in a sequence-dependent manner. This is clearly shown by the finding that PCR is inhibited by U-71184 only when the reaction is performed with primers amplifying the estrogen receptor region, but is not active in inhibiting the PCR-mediated amplification of the Ha-ras and FMR-1 genomic sequences (Figs. 3 and 6). In addition sequence-arrested experiments suggest that U-71184 binds to selected nucleotide sequences (data not shown). Accordingly, nucleotide sequences demonstrated by other groups to be recognized by U-71184 are present in the PCR-generated estrogen receptor fragment, such as ATAA, AATT, TTTA (Hurley et al., 1988, 1990; Aristoff, 1993). The same nucleotide sequences are not present within Ha-ras and FMR-1 PCR products (see Fig. 1).

As expected, the effects of U-71184 on PCR were found to be (i) similar to another A-T specific DNA binding drug, distamycin (Figs. 3 and 6) and (ii) sharply different from chromomycin, which recognizes G-C rich DNA sequences (Van Dyke and Dervan, 1983; Gao and Patel, 1989). Distamycin and chromomycin were selected as control DNA-binding drugs, since they have been well characterized by our group with respect to inhibitory effects on PCR (Passadore et al., 1994, 1995).

A second conclusion of the experiments reported in the present paper is that PCR performed on DNA from U-71184 treated cells is inhibited when the primers amplifying the ER region are used (Fig. 5). This approach might bring important information on both in vivo uptake of the drug by target cells and binding to DNA. This is a major point for pharmacological studies of DNA-binding drugs administered either to cells in culture or, more importantly, to experimental animals. In this respect, PCR can be used to determine whether U-71184 binds to genomic DNA of

cells belonging to selected tissues following in vivo administration.

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