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INHIBITION OF THE MOLONEY MURINE LEUKEMIA VIRUS CYCLE AT A POST REVERSE TRANSCRIPTIONAL STEP BY THE NETROPSIN-INTERCALATING HYBRID MOLECULE NETROPSIN–OXAZOLOPYRIDOCARBAZOLE

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Abstract—In a search for new antiretroviral agents acting at the nucleic acid level, two hybrid molecules composed of a bispyrrolecarboxamide chain related to netropsin, linked to the intercalating chromophore oxazolopyridocarbazole, were tested on the cycle of a defective Moloney murine leukemia virus (M.MuLV) derived from the SVX shuttle and expressing resistance to the G418 antibiotic. The drug netropsin–oxazolopyridocarbazole (Net-OPC), which displays a binding preference to duplex DNA containing A + T bases, inhibits the retroviral replicative cycle ($IC_{50} = 4.8\ \mu M$). In contrast, the related molecule (bis)pyrollecarboxamide-oxazolopyridocarbazole (Bpc-OPC) devoid of sequence preference as well as the elemental components of Net-OPC, namely OPC, pentyl-OPC and netropsin, displays no significant action on the viral cycle. The estimation of cytosolic viral DNA in infected cells using quantitative polymerase chain reaction suggests that Net-OPC impairs a post retrotranscriptional step of the viral cycle.

In an attempt to obtain compounds able to alter selectively genomic functions involving the recognition of A + T containing sequences [1], we have previously synthesized [2–4] netropsin-like conjugates in which the guanidine moiety of the netropsin molecule has been replaced by a tetramethylene chain linked to the intercalating chromophore oxazolopyridocarbazole (OPC§) [5, 6]. Among the different molecules synthesized, the compound displaying an oligopeptide chain identical to netropsin (i.e. bis-pyrrolecarboxamide-amidine moiety) (Net-OPC) (Fig. 1) was found to exhibit a markedly high selective affinity to poly d(A-T) and poly dA-dT. Footprinting experiments performed on a pBR322 DNA fragment have further shown that Net-OPC strongly protected the restriction endonuclease Eco RI site 3′-CTTAAG-5′ sequences [4]. In agreement with the binding to the Eco RI site, Net-OPC has been found to inhibit strongly and selectively the endonuclease activity of the enzyme [2, 4]. The efficiency of this inhibition was found to be markedly higher than that of netropsin. This finding indicates that DNA–protein complexes occurring at or in the vicinity of A + T-rich sequences can be considered as preferred targets for this ligand. Assuming that in infected cells, cytosolic retroviral DNA is preferentially accessible and that protein–DNA complexes occur naturally in the steps leading to integration into the host genome, we decided to investigate the effect of netropsin derivatives on this part of the Moloney murine leukemia virus (M.MuLV) replicative cycle. Thus, we comparatively investigated the effect of Net-OPC and the related molecule Bpc-OPC (Fig. 1) devoid of the sequence recognition property [2], on the M.MuLV replicative cycle. For this purpose, a method has been developed using the SVX murine shuttle vector [7] and taking advantage of the appearance in 3T3 cells of acquired resistance to the antibiotic G418 upon integration of recombinant retroviral DNA. Among the tested compounds, Net-OPC was found to inhibit selectively the viral cycle in host 3T3 cells. A possible effect of Net-OPC on the early stages of the infection process was further investigated by the quantification of the cytosolic viral DNA using a quantitative polymerase chain reaction (PCR) procedure.

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§ Abbreviations: OPC, oxazolopyridocarbazole; Net-OPC, netropsin–oxazolopyridocarbazole conjugate; Bpc-OPC, (Bis)pyrollecarboxamide–oxazolopyridicarbazole conjugate; M.MuLV, Moloney murine leukemia virus; PCR, polymerase chain reaction; AZT, 3′-azido-3′-deoxythymidine; LTR, long terminally repeated unit; CFU, colony forming unit.

MATERIALS AND METHODS

Chemicals

Polybren, obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.), was diluted in sterile NaCl (150 mM) to a concentration of 10 mg/mL. Geniticin G418 was from Gibco (Grand Island, NY, U.S.A.) and was stored at room temperature dissolved in sterile isotonic serum at a concentration of 30 mg/mL.

Synthesis of OPC, pentyl-OPC, Net-OPC and Bpc-OPC. Trimethyl-6*H*-[1,3]oxazolo[5,4-*C*]pyrido[3,4-*g*]-carbazole (OPC), 2-pentyl-7,10,12-trimethyl-6*H*-[1,3]oxazolo[5,4-*C*]pyrido[3,4-*g*]-carbazole (pentyl-OPC) and 2-pentanoic acid-7,10,12-trimethyl-6*H*-[1,3]oxazolo[5,4-*C*]pyrido[3,4-*g*]-carbazole (OPC-

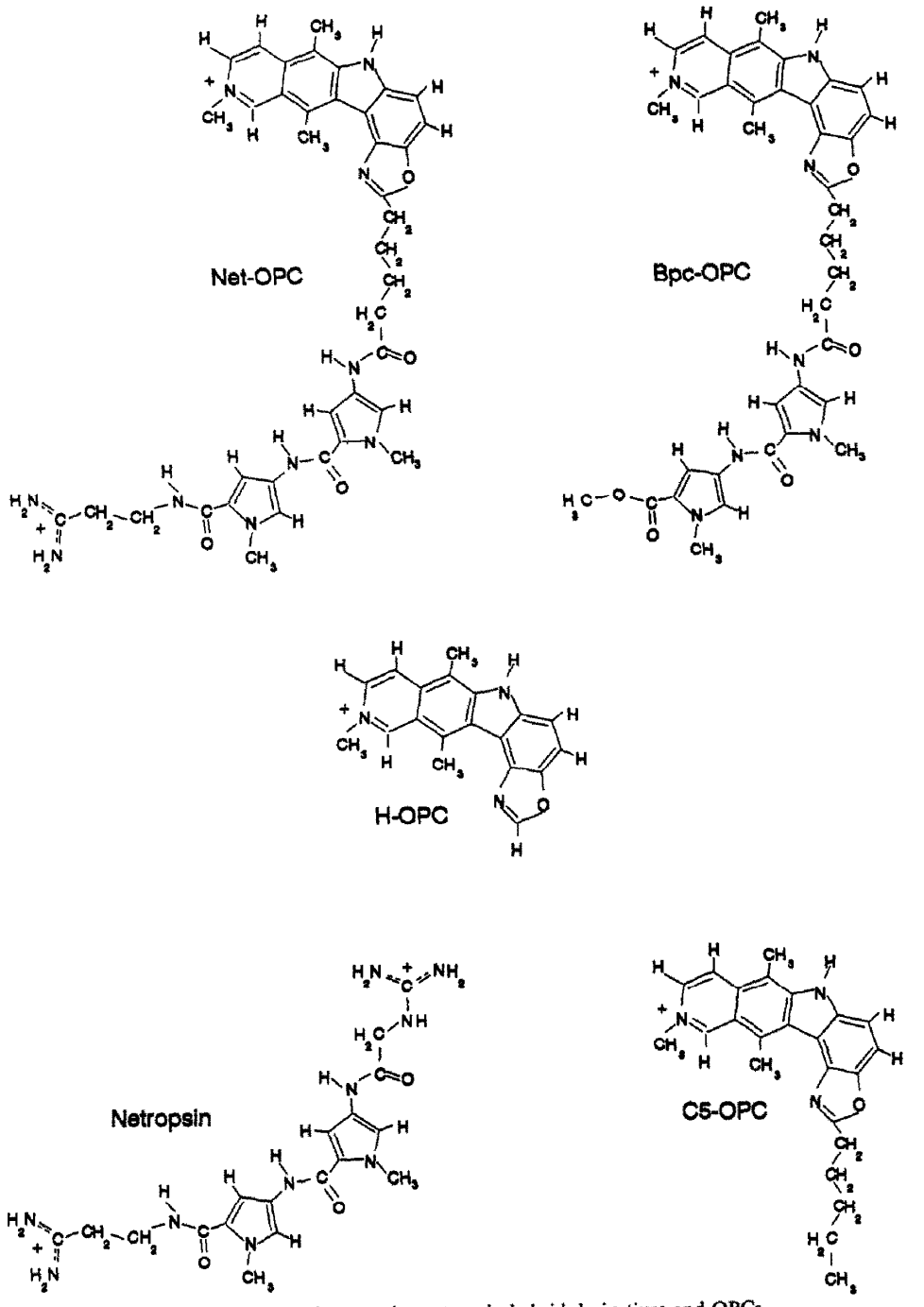

Fig. 1. Structure of netropsin, netropsin hybrid derivatives and OPCs.

valerate) were prepared from the antitumor agent 2*N*-methyl-9-hydroxyellipticinium (Celiptium) (NMHE) according to previously described procedures [5, 6]. Netropsin-OPC conjugates: 3-{1-methyl-4-[1-methyl-4-[4-(2-{7,10,12-trimethyl-6*H*-[1, 3]oxazolo[5, 4-*C*]pyrido[3, 4-*g*]carbazole})-butyl-carboxamido]pyrrole-2-carboxamido]-pyrrole-2-carboxamido}propionamidine bis(acetate) (Net-OPC) and methyl-1-methyl-4-{1-methyl-4[4-(2-{7,10,12-trimethyl-6*H*-[1,3]oxazolo[5,4-*C*]-pyrido [3,4-*g*]-carbazole})butylcarboxamido]pyrrole-2-carboxylate acetate (Bpc-OPC) were prepared as described previously [3, 4].

Cells and virus

Chronically infected cells (psi2 neo). The psi⁻2 cell lines were derived from 3T3 cells and contain, integrated in their cellular genome, the pMov-psi⁻

DNA that provides all the *trans* functions (*gag pol env* genes) required for the encapsulation of recombinant viral RNA [8]. The psi2 neo cell line was obtained by transfection of the SVX shuttle vector [7] in psi$^-$ 2 cells. SVX DNA contains the M.MuLV transcriptional unit, the *cis* acting region required for packaging of the viral RNA (i.e. the sequence psi) and encodes G418 resistance in mammalian cells. This genetic information leads to the replication and production of infectious defective viral particles containing the sequence psi and the *neo* gene. The psi2 neo cell lines used in the present work produce a titre of recombinant viruses varying from 10^4 to 10^6 CFU/mL. The NIH 3T3 and the psi2 neo cells were maintained in Dulbecco's modified Eagle medium (DMEM, Gibco Europe) containing 5% fetal calf serum, 100 μg/mL streptomycin and 100 U/mL penicillin.

M.MuLV retroviral cycle assay

Production of the defective M.MuLV recombinant. psi2 neo cells (5×10^5) were plated in 100-mm petri dishes and incubated at 37°. Twenty-four hours after plating, the supernatants containing the defective viral particles were harvested and filtered through a 0.45 μm Nalgen HA filter. The viral suspension was either used extemporaneously or aliquoted and stored at −70°. The number of infectious recombinant viral particles is estimated using the geniticin resistance assay as described below.

Infection of the 3T3 cells by the defective M.MuLV recombinant. NIH 3T3 cells (1.5×10^5) were plated in 60-mm petri dishes and incubated at 37°. Twenty-four hours after plating, 2 mL of an appropriate dilution of a viral suspension containing 8 μg/mL polybren were added to the petri dishes containing the 3T3 cells. In the standard operating conditions, the drugs to be tested were added together with the viral suspension used to infect the 3T3 cells. Twenty-four hours after the infection, geniticin G418 was added to a concentration of 0.6 mg/mL. Six days later the resistant clones were colored using violet crystal dye and counted (Fig. 2B). In these conditions the number of resistant clones is a linear function of the number of infectious viral particles added, as shown in Fig. 2A. The possible cytostatic or cytotoxic effect of the tested drugs were estimated as follows: cytotoxicity control dishes in which geniticin was omitted were monitored in parallel. Twenty-four hours following the infection, the cells were trypsinized and counted. Aliquots of 300 and 500 cells were then replated and the colonies counted 6 days later.

Preparation of the cytosolic fractions. In this experiment, 10^6 NIH 3T3 cells in DMEM containing 5% calf serum were plated in 100-mm petri dishes. Twenty-four hours after plating, the cells were infected by addition of 10 mL of an appropriate dilution of viral suspension. Drugs to be tested were added together with the viral suspension used to infect the cells. Twenty-four hours following the infection, the cells were harvested by trypsinization and treated essentially as described by Brown *et al.* [9]. Briefly, the cells were washed with buffer A (pH 7.4) composed of 10 mM Tris–HCl, 225 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol and 20 μg/mL aprotinine. The cells were lysed using a mixture of 250 μL buffer A containing 0.025% digitonin. The lysate was centrifuged at 1000 *g* for 3 min. The supernantant was then centrifuged at 9000 *g* for 20 min. The supernatant was referred to as the cytosolic extract. This fraction contains full length viral DNA as well as subgenomic intermediates. After incubation overnight in the presence of 0.5% SDS and 50 μg/mL proteinase K, the cytosolic DNA was extracted by phenol/chloroform, precipitated by ethanol and washed with a 70% ethanol solution. Purified DNA was dissolved in Tris–HCl buffer (pH 7.6) containing 1 mM EDTA.

Amplification of the neo *gene fragment by PCR.* The amplification was carried out using an automated temperature device (Cetus). Five hundred and eighty base pairs of the *neo* gene were amplified using the following primers: neo1: 5′-GGCTATTCGGCTATGACTGG (melting temperature of the corresponding duplex 62°) and neo2: 5′-CGGCCACAGTCGATGAATCC (melting temperature 64°). The oligonucleotide primers were synthesized using a Biosystem 381A automatic synthesizer according to standard operating conditions and purified by polyacrylamide (15%) gel electrophoresis. In the standard conditions, the amplification reaction involves one step of precycle (94°, 2 min) followed by 25 cycles of 62°, 1 min and 75°, 30 sec and 94°, 1 min. The termination of the elongation involves one step 62°, 1 min followed by 75°, 10 min. After the amplification steps, aliquots of the reaction mixtures were subjected to polyacrylamide (8%) gel electrophoresis for 3 hr and the bands of amplified DNA were stained with ethidium bromide. The density of the bands was measured using a Joyce-Loebl chromoscan III densitometer.

Reverse transcription assays. Assays were performed in a reaction mixture containing 50 mM Tris–HCl pH 7.6, 30 mM NaCl, 10 mM $MnCl_2$, 20 mM dithiothreitol. The templates/primers used were either poly dC/oligo dG or poly dA/oligo dT. dGTP (μM) and 1 μCl [^{3}H]dGTP or 16 μM TTP and 1 μCl [^{3}H]TTP were added when using, respectively, poly dG/oligo dC or poly dA/oligo dT. The reaction was started by the addition of 10 μL of 50 times concentrated psi2 neo supernatant. Assays were carried out at 37° for 3 hr and the entire mixture was spotted onto Whatman GF/C filters. The filters were washed three times in ice-cold TCA 10%, three times more in TCA 5% and finally in cold ethanol. The papers were dried and acid-insoluble radioactivity was subsequently counted.

RESULTS AND DISCUSSION

Inhibition of M.MuLV cycle by Net-OPC

In order to validate the methodology described in the methods in view of drug testing, we verified that under our experimental conditions, the treatment of the infected 3T3 cells by non-cytotoxic concentrations of the reverse transcriptase inhibitor 3′-azido-3′-deoxythymidine (AZT) results in the inhibition of the production of G418 resistant clones (Fig. 3). We then tested the effect of the two related hybrid molecules on the viral cycle, namely the netropsin-

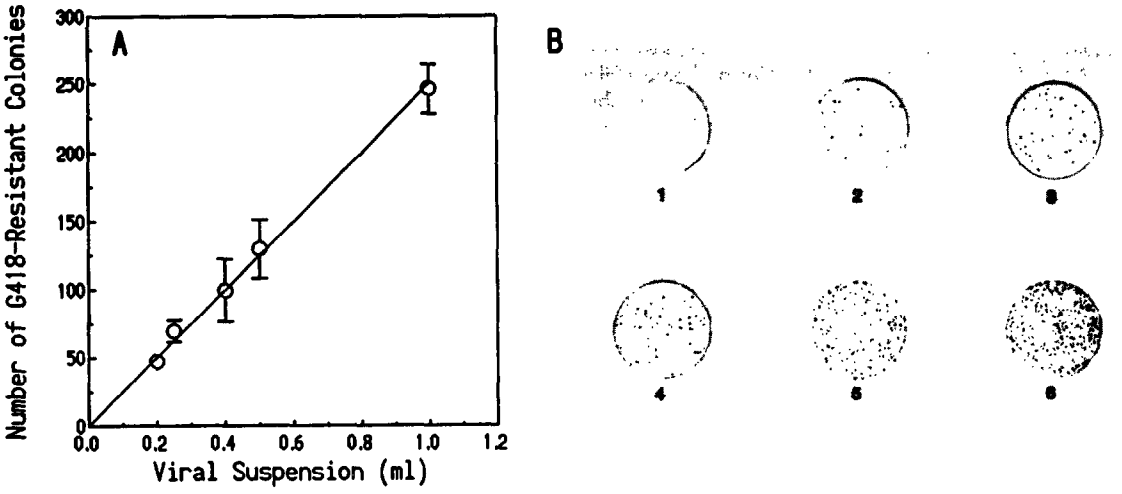

Fig. 2. Effect of the number of recombinant viruses on the formation of G418-resistant 3T3 clones. The Psi2 neo cell line used in the present work produces a titer of recombinant viruses of 10^6 CFU/mL. Psi2 neo cells (5×10^5) were plated with 7 mL of medium, incubated at 37° and further treated as indicated in Materials and Methods. In these conditions, the number of resistant clones is a linear function of the number of infectious viral particles added (A). The values indicated are the mean ± SD of three different experiments. (B) Example of the formation of G418-resistant colonies upon infection of cells by the recombinant virus. Plate 1, no infection; plates 2–6, infection of 3T3 by increasing amounts of virus (1, no virus; 2, 2×10^5 CFU; 3, 2.5×10^5 CFU; 4, 4×10^5 CFU; 5, 5×10^5 CFU; 6, 10^6 CFU).

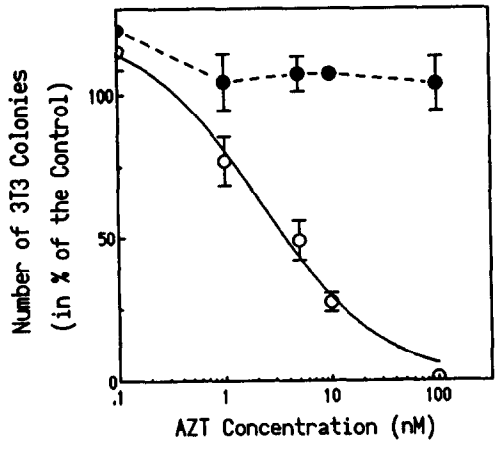

Fig. 3. Effect of AZT on the formation of G418-resistant clones in 3T3 cell populations infected by the M.MuLV recombinant. Experiments were performed using the standard operating conditions described in the legend of Fig. 2. Infection of 3T3 was performed using 0.4 mL of viral suspension yielding about 100 resistant colonies per plate. The drug to be tested (AZT obtained from Retrovir capsules, Wellcome) was added together with the viral suspension. The possible cytotoxic effect of the tested drug was estimated as follows: cytotoxicity control dishes were monitored in parallel as described above except that G418 was omitted. Twenty-four hours after the infection, the cells were trypsinized and counted. Three hundred cells were then plated and the colonies counted 6 days later. (○) Indicates the infection assay and (●) the cytotoxic assay.

intercalating drug Net-OPC, and Bpc-OPC which in comparison with Net-OPC lacks the positively charged amidinium side chain (Fig. 1). The results in Fig. 4A show that the addition of increasing concentrations of Net-OPC in a medium containing 3T3 cells and infectious defective M.MuLV results in a decrease ($ID_{50} = 4.8\ \mu M$) and a complete disappearance of G418-resistant clones. It was verified that in the range of concentrations used, the drug did not induce either cytostatic or cytotoxic effects on infected 3T3 cells. In order to rule out a possible synergistic toxic effect of Net-OPC and geniticin we tested the effect of Net-OPC on resistant 3T3 cells (displaying M.MuLV provirus in their genome) in the presence of G418. No toxic effect was observed in these conditions (data not shown). In contrast to the effect of Net-OPC, the addition of the same concentrations of Bpc-OPC in the culture medium results in a smaller decrease in the number of resistant clones (Fig. 4B) and also in a cytotoxic effect on the 3T3 cells. The toxicity curve is more or less superimposed on the curve describing the decrease of the production of neo-resistant cells. When corrected for the cytotoxic effect (insert in Fig. 4B), no significant selective inhibition of the production of neo-resistant cells remains visible. Table 1 summarized the effect of the molecular components of Net-OPC, including OPC, pentyl-OPC and netropsin, on the appearance of G418-resistant 3T3 colonies. Clearly, Net-OPC only displays a selective effect on the viral cycle. The absence of an effect of netropsin on both the viral cycle and the viability of the host cells is probably due to an unfavorable diffusion of the drug across the 3T3 cell membrane.

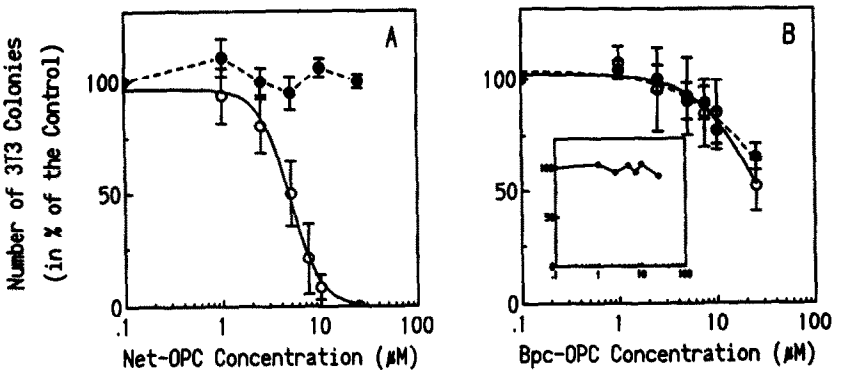

Fig. 4. Comparative effect of Net-OPC and Bpc-OPC on the formation of G418-resistant clones in 3T3 cell populations infected by the M.MuLV recombinant. (A) (●) Represents the cytotoxic effect of Net-OPC as measured by the plating efficiency and (○) the number of G418-resistant colonies. (B) (●) Represents the cytotoxic effect of Bpc-OPC as measured by the plating efficiency and (○) the number of G418-resistant colonies. The insert indicates the effect of Bpc-OPC once corrected for cytotoxicity. Data are the mean ± SD of at least three different experiments. All experiments were performed using the standard operating condition as described in the legend of Fig. 3. In this experiment, it was also verified that the tested drugs did not inhibit the expression of the *neo* gene using 3T3 cells displaying viral DNA integrated in their genome.

Table 1. Inhibition of the formation of G418-resistant colonies and cytotoxic activity of Net-OPC and related molecules

Compound	Inhibition of the formation of resistant colonies ED_{50} (μM)	Cytotoxicity
Netropsin	NE	NT
Net-OPC	4.6	NT
Bpc-OPC	29.2	39.6
Pentyl-OPC	3.5	5.0
OPC	50.0	69.0

The formation of G418-resistant 3T3 colonies as well as the cytotoxic effect of the tested molecules were estimated as described in Materials and Methods. The ED_{50} corresponding to the drug concentration which gives 50% reduction of either the number of G418-resistant colonies or the cloning efficiency of 3T3 cells was calculated from the curves shown in Fig. 4 using non-linear regression fitting.

NE, no effect.

NT, non-toxic.

Effect of Net-OPC on viral particle internalization and viral RNA reverse transcription

It should be noted that the decrease in the number of G418-resistant clones observed in the presence of increasing concentrations of Net-OPC and shown in Fig. 4A may result from the inhibition of one of the steps of the retroviral cycle, including the internalization of the virus in 3T3 cells, the reverse transcription of viral RNA to viral DNA and the integration process. In order to obtain additional information concerning the nature of the step impaired by treatment with Net-OPC, we investigated the effect of the drug on the amount of viral DNA present in the cytosol of infected 3T3 cells, which should depend on the efficiency of both the internalization of the virus inside the cells and the reverse transcriptase activity. For this purpose the viral DNA was quantified by the amplification of a part of the *neo* gene present on the viral DNA using a quantitative PCR procedure. Data in Fig. 5A and C show that the treatment of the cells by AZT used as a control drug results, as expected, in a marked decrease and complete disappearance of the viral cytosolic DNA. In contrast, the treatment of the cells by Net-OPC using concentrations which inhibit the production of G418-resistant clones did not result in any significant modification of the amount of cytosolic viral DNA (Fig. 5B and D), indicating that the drug did not act on the penetration of the viral particles and suggesting that the reverse transcription of the viral RNA is not impaired. We verified that the addition of increasing concentrations of Net-OPC up to 50 μM did not alter the reverse transcriptase activity in the *in vitro* assay system (see Materials and Methods) containing artificial template/primer poly-rC/oligo-dG or poly-rT/oligo-dA (data not shown). Nevertheless, it cannot be excluded that a non-integrative sub-genomic form of viral DNA could be generated. Despite this later possibility, it appears equally reasonable to hypothesize that Net-OPC could act either on the migration of viral DNA from the cytosol to the cell nucleus or at the integration step of the viral DNA into the host genome. This later hypothesis appears more likely for the following reasons:

The integration process of retroviruses has been shown to require the integration protein which binds to the linear viral DNA att sites consisting of short imperfect inverted repeat sequences located at both ends of the viral DNA within the long terminally

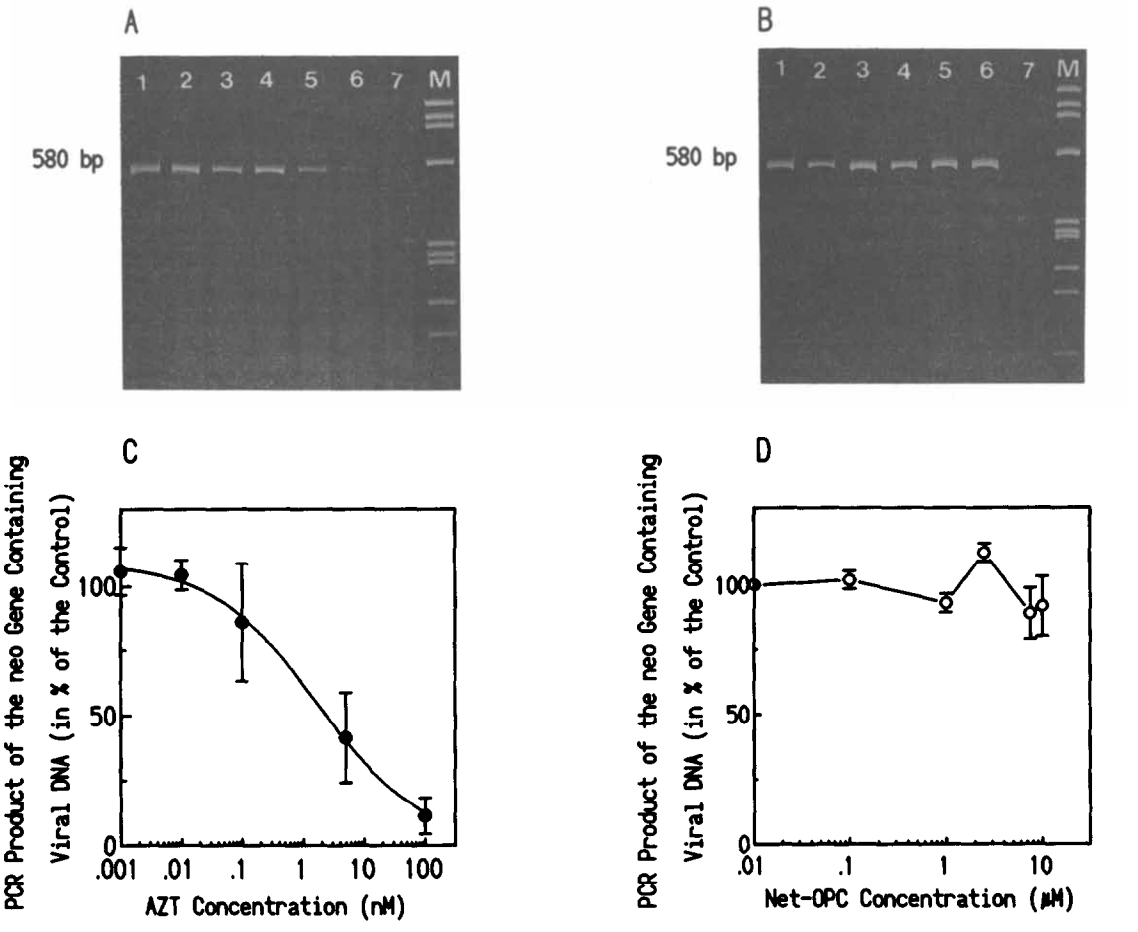

Fig. 5. Effect of AZT (A, C) and Net-OPC (B, D) on the production of recombinant viral DNA in 3T3 infected cells as detected by PCR amplification. Twenty hours after infection of the 3T3 cells by the defective M.MuLV, the cells were harvested by trypsinization and the cytosolic fractions were prepared as described in Materials and Methods. (A, B) Samples of cytosolic fractions (3 μg DNA) were amplified by PCR as described in Materials and Methods. Lanes 1–6: amplification of the *neo* gene fragment (580 bp) extracted from the cytosolic fractions of cells treated with increasing concentrations of AZT or Net-OPC. Lane 7: amplification of the cytosolic fraction from non-infected 3T3 cells. In the standard conditions, the amount of viral DNA in the cytosol roughly corresponds to one copy per cell. Lane M: Phage phi-X174 fragments resulting from HaeIII digestion were used as molecular mass markers. (C, D) Semi-logarithmic representation of the PCR products as a function of AZT or Net-OPC concentration.

repeated units (LTRs) [10]. The terminal 13 bp located at the ends of the LTRs are sufficient for binding of the integration protein and are required for efficient integration [11]. *In vitro* experiments using model integration substrates [12–14] have shown that 3′-ACTTTCT-5′ was the sequence within the 13 bp that was primarily responsible for correct integration.

Furthermore, in connection with the binding preference to A + T-containing sequences, footprinting experiments performed on a pBR322 DNA fragment have shown that Net-OPC strongly protected the restriction endonuclease Eco RI site 3′-CTTAAG-5′ as well as 3′-ACTT and 3′-CTTT regions [4]. The 3′-ACTTTC sequence of the LTR of the viral DNA therefore appears to be a good candidate for the target of Net-OPC.

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